Abstract

Mutagenic Potential of Nickel Compounds in Cultured Human Lymphoblasts *In Vitro*

The mutagenic potential of nickel sulfate, nickel subsulfide, nickel hydroxide, nickel oxide (green), and nickel metal powder was determined in cultured TK6 human lymphoblasts at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) and thymidine kinase (*tk*) loci. TK6 cells were treated with nickel compounds for 24 hours at several cytotoxic concentrations. All of the nickel compounds tested failed to induce *hprt*$^-$ mutants and failed to induce normal-growing *tk*$^{-/-}$ mutants. These results indicate that the nickel compounds do not efficiently induce base pair substitutions, frameshift mutations, or small deletions in human cells. However, nickel sulfate, nickel subsulfide, nickel hydroxide and nickel metal powder induced slow-growing *tk*$^{-/-}$ mutants. This phenotype has been previously shown to be associated with large DNA alterations in the *tk* gene. Significant induction of slow-growing *tk*$^{-/-}$ mutants occurred only at nickel concentrations that were cytotoxic. No significant increase in the frequency of slow-growing *tk*$^{-/-}$ was observed with nickel oxide.

Work by others has demonstrated that nickel can catalyze oxidative damage of DNA bases in the presence of hydrogen peroxide (H_2O_2) *in vitro*. Therefore, the effect of added H_2O_2 on the mutagenicity of these nickel compounds was also investigated. TK6 cells were exposed to nickel compounds for 8 hours to permit uptake, and then exposed for an additional 16 hours following the addition of 20 uM H_2O_2; this concentration of H_2O_2 resulted in measurable toxicity and mutagenicity in TK6 cells. The addition of H_2O_2 shifted the dose-response relationship for nickel-induced slow-growing *tk*$^{-/-}$ mutants to lower nickel concentrations, but the effect was less than a factor of two. To explore further the possible role of reactive oxygen species in nickel mutagenesis, TK6 cells were treated with nickel in the absence and presence of the radical scavenger vitamin E (25 uM) . No significant decrease in the mutation frequency was observed with vitamin E. These results suggest that the induction of large DNA alterations in human cells by nickel compounds may not be mediated by oxygen radicals.

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List of Abbreviations

CFU	Colony forming units
CHAT	Cytidine, Hypoxanthine, aminopterin, thymidine
CHO	Chinese hamster ovary cells
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethane
HPRT	Hypoxanthine-guanine phosphoribosyltransferase (*hprt*)
IMF	Induced mutant fraction (mutant fraction minus spontaneous background)
Ni(II)	Ionic nickel (Ni^{+2})
Pen/Strep	Penicillin-streptomycin
RPMI	RPMI 1640 + L-glutamine
SHE	Syrian hamster embryo cells
TFT	Trifluorothymidine
6-TG	6-Thioguanine
THC	Cytidine, hypoxanthine, thymidine
TK	Thymidine kinase (*tk*)
TK6	Human lymphoblastoid TK6 cell line
NORMAL-GROWING *TK*$^{-/-}$ MUTANTS	Trifluorothymidine resistant mutants that have a normal growth rate (14-18 hrs) relative to wild-type TK6.
SLOW-GROWING *TK*$^{-/-}$ MUTANTS	Trifluorothymidine resistant mutants that have a slow growth rate (21-44 hrs) relative to wild-type TK6.
8-OH-dG	8-hydroxy-2'-deoxyguanosine

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1. Introduction

Certain forms of nickel have been associated with increased occurrence of nasal and lung cancer in humans based on epidemiological studies of refinery workers (Morgan, 1958; Doll et al., 1977). Nickel has also been shown to induce tumors in a wide variety of species (Sunderman, 1984; US EPA, 1986). *In vitro* studies of mammalian and human cells indicate that nickel is capable of causing several types of DNA damage, including DNA strand breaks, sister chromatid exchanges, DNA-protein cross-links, and chromosome aberrations (Ciccarelli et al., 1981; Sen and Costa, 1986, Nishimura and Umeda, 1979). Nickel has also been shown to induce mutations and cellular transformation in several test systems (Miyaki et al., 1979; Costa et al., 1989; Hansen and Stern, 1984; Swierenga and McLean, 1985).

Despite the extensive research that has been conducted, the mechanism by which nickel exerts genotoxic and mutagenic effects remains unknown. Ni(II) only binds weakly to DNA in solution, and this interaction causes little or no direct DNA damage (Eichhorn et al., 1968). Ni(II) has also been shown to have a higher affinity for proteins than DNA in chromatin suggesting that proteins may play a role in nickel-induced mutagenesis (Ciccarelli and Wetterhahn, 1984). Oxygen free-radicals have also been implicated in Ni(II)-induced DNA damage by the oxidation of DNA bases (Nackerdien, et al. 1991). Collectively, this evidence suggests that nickel may act on DNA indirectly by causing damage through intermediates, such as oxygen radicals, or by inhibiting enzymes responsible for DNA replication or repair.

A logical starting point in the investigation of any chemical carcinogen is a rigorous test of its mutagenic potential *in vitro*. In addition, establishing the molecular mechanism by which Ni(II) induces DNA damage and mutation is essential to understanding the association between nickel and cancer and to improve cancer risk assessment. Knowledge of the mechanisms helps facilitate extrapolation of results from *in vitro* assays to animal and epidemiological studies, and to extrapolate across different species. Improved understanding of the mechanism also helps to establish whether a threshold level exists for nickel.

A definitive study of the mutagenicity of nickel has not been carried out previously in human cells. The specific aims of this research are to: (i) determine the mutagenic potential of nickel subsulfide (Ni_3S_2), nickel oxide (NiO-green), nickel hydroxide [$Ni(OH)_2$], nickel sulfate ($NiSO_4$), and nickel metal powder (Ni) in the TK6 human lymphoblastoid cell line at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) and thymidine kinase (*tk*) loci, (ii) investigate whether reactive oxygen species produced from added hydrogen peroxide (H_2O_2) are involved in nickel-induced mutagenesis, and (iii) test for the inhibition of mutation by a radical scavenger for the nickel compounds that induced a mutagenic response.

2. Literature Review

2.1 Chemical and Physical Properties of Nickel

Nickel is a ubiquitous trace metal that can be detected in all parts of the environment, including plants and animals consumed by humans, air, drinking water, rivers, lakes, oceans, and soil. It comprises approximately 0.008% of the earth's crust and 8.5% of the earth's core (Nriagu, 1980). Nickel is often a component of silicate, sulfide, or arsenide ores, and is a constituent of most meteorites. In the environment and biological systems, nickel is most commonly found as Ni^{+2} [Ni(II)], however, it may also be found in several other oxidation states (-1,+1,+3,+4). Nickel is hard, malleable, ductile, somewhat ferromagnetic, and a conductor of heat and electricity. As a transition metal, nickel is uniquely resistant to alkalis, but generally dissolves in dilute oxidizing acids.

Nickel compounds vary significantly in their chemical and physical properties (Table 1). The compounds used in the present study, for example, range from highly water-soluble (nickel sulfate) and soluble in biological media over several days (nickel subsulfide, nickel hydroxide), to completely insoluble with dissolution rates of greater than eleven years (nickel metal powder, nickel oxide). Nickel compounds also vary in other characteristics including density, melting temperature, and physical appearance.

2.2 Uses of Nickel

Nickel is a valuable mineral because of its resistance to corrosion and its ability to form nickel-iron alloys. Stainless steel is the most well-known alloy; others include permanent magnet and super alloys which are used in radios, generators and turbochargers, and copper-nickel alloys which are known for resistance to extreme stress and temperature. Tubing made of copper-nickel alloy is extensively used in making desalination plants. Nickel is also used for making spark plugs, alkaline (nickel/cadmium) batteries, coins, pigments, and is a catalyst for the hydrogenation of vegetable oils.

2.3 Sources of Nickel

Nickel is obtained commercially from many parts of world, including the former USSR, Canada, Japan, Norway, Australia, and New Caledonia (Table 2). A large resource of yet untapped nickel is in the seabed (ICPS, 1991).

Nickel is emitted into the environment from both natural and anthropogenic sources and is circulated throughout all environmental compartments. Natural sources of atmospheric nickel include: windblown dust, volcanic ash, vegetation, forest fires, meteoric dust, and ocean sea spray. Anthropogenic sources also make a significant contribution to nickel emissions including: the combustion of fossil fuels, nonferrous metal production and municipal waste incineration.

Nickel is also released into the hydrosphere and soil by the chemical and physical degradation of rocks, by atmospheric deposition, and by a variety of anthropogenic sources, including: industrial discharges, storm water runoff, and waste water and sewage sludge disposal (US EPA, 1986). The levels of nickel in the environment may vary significantly from remote to urban and industrial areas, and in some locations, nickel from natural sources has been reported to exceed anthropogenic inputs (Niragu, 1980).

2.4 Environmental Cycling of Nickel

Atmospheric nickel primarily exists in the form of particulate aerosols that contain different concentrations of nickel depending on the source. The transport and distribution of nickel particles between environmental compartments is strongly influenced by particle size and meteorological conditions (ICPS, 1991).

In lakes and rivers, nickel is mainly associated with organic matter that may be transported to the sediments or absorbed by biota. Nickel may be released from the sediment and recirculated throughout a body of water, particularly during periods of high turbulence or seasonal turnover.

Nickel also exhibits high mobility in the soil, facilitating its transport to groundwater, rivers and lakes. Acid rain is known to release nickel from the soil for transport into other environmental compartments (US EPA, 1986). Terrestrial plants and wildlife take up nickel, however, there is little evidence for accumulation or biomagnification of nickel in the food chain (ICPS, 1991).

2.5 Exposure to Nickel

2.5.1) Environmental Exposure

Concentrations of nickel in the environment may vary in time and space, over several orders of magnitude because of the large number of sources releasing nickel and their uneven distribution about the globe.

In the atmosphere urban nickel concentrations, enhanced by an abundance of anthropogenic emission sources, generally fall between 20 and 80 ng/m^3 with some extreme values over 200 ng/m^3 (Schmidt and Andren, 1980). Nickel concentrations are lower in rural and semi-urban environments which are more removed from anthropogenic sources (1-10 ng/m^3). In extremely remote areas, nickel levels in air drop off dramatically to levels that are largely due to soil suspension (<0.1 to 3 ng/m^3) (Schmidt and Andren, 1980). The predominant nickel compounds in the atmosphere include nickel sulfate, oxides, and sulfides, and to a lesser extent, metallic nickel. (IPCS, 1991). Overall, mean ambient nickel concentrations in the US atmosphere appear to have declined from 0.012 ug/m^3 in 1977 to 0.008 ug/m^3 in 1982 (US EPA, 1986).

In aquatic systems nickel concentrations are generally low unless they are impacted by industrial discharges. Nickel concentrations in the US usually vary from 3.0 to 17.0 ug/l for fresh water and from 0.1 to 0.5 for sea water (Jenkins, 1980). Mean nickel concentrations in 15 major river basins in the continental US ranged from less than 5 ug/l to greater than 700 ug/l between 1980 and 1982 (US EPA, 1986). The Ohio River basin consistently shows the highest mean nickel concentration, ranging from 552 ug/l in 1980 to 672 ug/l in 1982 (US EPA, 1986). The maximum reported concentrations for this basin were between 7,800 and 10,900 ug/l (US EPA, 1986). Effluents from waste-water treatment plants may also have a high nickel content containing up to 0.2 mg/l (ICPS, 1991).

Nickel concentrations in soil and vegetation may also vary considerably. Natural soil levels range from 5 to 500 ppm, however, soils derived from serpentine rock or agricultural land amended with sewage sludge may contain higher levels of nickel from 10 to 53,000 ppm (ICPS, 1991). The US Environmental Protection Agency estimates that the average level of nickel in soil is approximately 50 ppm (US EPA, 1986). For cultivated crops and natural vegetation nickel is usually below the 1 ppm level with concentrations ranging from 0.05 to 5 ppm (US EPA, 1986).

2.5.2) Occupational Exposure

Nickel concentrations may be significantly higher in the working environment than normal atmospheric air levels (Mastromatteo, 1986, 1988). In 1977 the US National Institute of Occupational Safety and Health (NIOSH) estimated that approximately 250,000 individuals in the US were exposed to nickel in a wide variety of occupations (US NIOSH, 1977) (Table 3). NIOSH reported concentrations ranging from a few ug/m^3 to several mg/m^3 for workers in smelting, refining, alloy production, welding, and other processes. Concentrations of nickel in the work environment can vary considerably, depending upon the type of operation, the concentration of the material being handled, and proper ventilation. For example, in alloy production an average concentration of airborne nickel during picking and handling was determined to be 0.008 mg/m^3, whereas, during grinding, the average airborne nickel level was 0.298 mg/m^3 (Warner, 1984). Nickel workers may also be exposed to other metals or chemicals including arsenic, chromium, cobalt, and benzo[a]pyrene (ICPS, 1991).

Improvements in operational techniques and ventilation over the last several decades have significantly reduced nickel concentrations in the work environment. Average concentrations in the US are maintained well below the permissible exposure limit (PEL) of 1 mg/m^3 set by the Occupational Safety and Health Administration (OSHA) (US EPA, 1986). Workers in developing countries, however, may lack proper safety regulations and can subsequently be exposed to much higher nickel concentrations.

2.5.3) Routes of Exposure and Intake

The major routes of nickel exposure to humans include inhalation, ingestion, and percutaneous absorption. Parenteral administration of nickel is of interest in

experimental studies and is particularly helpful in evaluating the kinetics of nickel transport, distribution and excretion. Transplacental transfer to the fetus is also important in the assessment of *in utero* effects.

Segments of the population that may be exposed to high levels of nickel include people whose diets contain foods naturally high in nickel, people who are occupationally exposed to nickel, people living in the vicinity of a nickel processing facility and people who smoke tobacco. For the general population, dietary intake is often the highest level of nickel exposure because it is found in most foods--including fruits, vegetables, grains, seafood and breast milk. Average daily nickel intake is estimated at 160 ug/day (Sigel, 1988), however intake levels may vary widely from 100 to 800 ug/day as a function of dietary habits (Clemente and Rossi, 1980). Certain types of foods, such as soya beans and cocoa, have been found to be especially high in nickel content (5.20 and 9.80 ppm nickel, respectively) and may lead to elevated nickel exposures (US EPA, 1986). Dietary nickel intake may also be increased by the use of certain fertilizers on food crops or by the use of nickel-containing cooking vessels or utensils.

Inhalation is an important route of exposure particularly for occupationally exposed people and tobacco smokers. Nickel may enter the respiratory tract in the form of an aerosol, dust, or gas. Particle deposition in the lung is highly dependent upon the size of the particle, with smaller particles reaching lower lung generations. An appreciable amount of nickel dust that is inhaled may be removed via the mucociliary clearance mechanism and swallowed, while other particles may be phagocytized and absorbed from the lung into the blood. Tobacco smoking significantly increases the amount of nickel inhaled and damages the mucociliary clearance mechanism that helps remove nickel from the lung. Individuals who smoke 40 cigarettes per day have been estimated to inhale between 2-23 ug nickel per day (ICPS, 1991).

Humans are also exposed to nickel in drinking water. Daily intake levels are typically less than found in the diet, however, nickel intake may be significantly increased by the leaching of nickel-containing plumbing or faucets. The average concentration of nickel in US water samples at the consumer's tap was 4.8 ug/l determined by sampling 969 US water supplies from 1965 to 1970 (ICPS, 1991).

A relatively minor route of exposure is dermal contact with a variety of nickel-containing consumer products, including kitchen utensils, jewelry, buttons, zippers, coins, medical,

and dental devices. Dermal exposure is commonly associated with contact dermatitis in nickel-sensitized individuals. Prostheses and other surgical devices, such as heart valves, implanted pacemakers, and intrauterine contraceptive devices, also may contain nickel that cause allergic skin reactions.

2.6 Nickel Toxicokinetics

2.6.1) Absorption

Nickel is primarily absorbed through the respiratory tract and gastrointestinal tract, although some nickel compounds may also penetrate the skin. In addition, iatrogenic exposure can occur due to surgical or dental implants and hemodialysis (Granjean, 1984). Absorption of nickel is dependent on the route of exposure, the physical and chemical characteristics of the compound, and biological factors, such as nutritional and health status of the exposed individual.

In the respiratory tract, soluble nickel compounds in the form of gases and aerosols pass through biological barriers more easily than particulate compounds (US EPA, 1986). Insoluble nickel compounds, such as nickel oxide and subsulfide, may accumulate over time in the lung (US EPA, 1986). Experimental animal data in respiratory tract of hamsters and rats show slow clearance of insoluble nickel oxide (half-time years) and relatively rapid clearance of soluble nickel salts (half-time hours to several days) (Wehner and Craig, 1972; Medinsky et al., 1987). In the nasal mucosa of nickel workers, nickel oxide has been shown to have a clearance half-time of approximately 3.5 years (Nieboer et al., 1988). Absorption of particulates in the lung is highly dependent upon particle deposition and clearance. Clearance may involve direct absorption into the blood stream or the lymphatic system, or removal via the mucociliary blanket.

Absorption of nickel in the gastrointestinal tract is low compared to the high dietary intake of most individuals. Collectively, data indicate that only 1 to 10% of dietary nickel is absorbed through the gastrointestinal tract (US EPA, 1986). As a result, this route of exposure is generally not associated with significant elevations in nickel body burden.

2.6.2) Transport and Distribution

The transport and distribution of nickel in an organism is influenced by several factors--including the mode of absorption, the rate and levels of exposure, the chemical/physical properties of the compound, and the physiological status of the host. Blood is the main vehicle for the transport of absorbed nickel to tissues throughout the body. In unexposed individuals, mean serum values are approximately 2.0 to 3.0 ug/l (US EPA, 1986). In plasma approximately 75% of nickel is bound to higher molecular weight proteins such as alpha-2-macroglobulin, gamma-globulin, transferrin, and albumin (Nomoto, et al., 1971; Van Soestbergen et al., 1972; Asato et al., 1975). Serum concentrations of human serum albumin and free amino acids (especially histidine) have been shown to exert a regulating influence on the cellular accumulation of nickel *in vitro*.

The distribution of nickel in the organs of exposed animals has been documented by a number of investigators (Table 4). Although differences in distribution occur as a function of both route of exposure and time after exposure, the kidney and lung are consistently the primary organs where nickel accumulation occurs. Placenta has been shown to have a high affinity for nickel in pregnant animals, and therefore does not act as a barrier protecting the fetus from nickel exposure. However, the placenta does appear to delay fetal exposure. Relatively little nickel is retained in neural tissue, consistent with the observed low neurotoxic potential of divalent nickel salts. Similarly, there is little retention of nickel in bone or mineral tissue (for review: see Coogan et al., 1989).

2.6.3) Cellular Uptake and Bioavailability

Nickel uptake occurs through three primary mechanisms: (i) endocytosis of particulate nickel compounds and alloys (ii) transport of soluble Ni(II) ions via Ca(II) channels, and (iii) transmembrane diffusion of lipophilic nickel compounds (e.g. nickel carbonyl) (Sunderman, 1989). The mode of uptake, as well as the bioavailability of the nickel compound significantly effect the ability of nickel to traverse the cellular membrane and be delivered to a critical target site (DNA or nuclear protein).

In the nickel ion hypothesis, the nickel ion [Ni(II)] or some other dissolution product is thought to be the ultimate carcinogenic form of nickel since particulate nickel cannot

enter the nucleus (Costa et al., 1981). The ion's intracellular concentration is considered the major determinant of effect, irrespective of the nickel compound to which an organism is exposed. DNA lesions caused by nickel compounds have been shown to correlate with the concentration of Ni(II) in the nucleus (Ciccarelli and Wetterhahn, 1982).

The phagocytosis of particulate nickel has been observed by electron microscopy and by video-intensification microscopy (Costa et al., 1981; Evans et al., 1982). Nickel particles are engulfed by the cell and stored in cytoplasmic vacuoles which accumulate around the periphery of the nucleus. The vacuole's acidic environment facilitates particle dissolution into nickel ions that are capable of traversing the nuclear membrane and binding to DNA (Evans et al., 1982; Abbracchio et al., 1982a).

The carcinogenicity of particulate nickel compounds was found to be directly proportional to the rate of cellular uptake in *in vitro* cellular test assays (Costa and Mollenhauer, 1980). Crystalline nickel monosulfide, which is a potent carcinogen, is actively phagocytized by cultured fibroblasts *in vitro*; whereas amorphous nickel monosulfide, which is noncarcinogenic, has a relatively slow rate of uptake (Abbracchio et al., 1982b; Costa et al., 1980). Differences in the phagocytic uptake and the transforming capability of these two compounds was observed to be related to the surface charge on the nickel particles, with uptake favoring the negatively charged crystalline NiS. Further studies confirmed that treatment of amorphous nickel monosulfide with a strong reducing agent ($LiALH_4$), gave the particle a negative surface charge, facilitating phagocytosis and increasing cellular transformation (Heck and Costa, 1982).

Kuehn and Sunderman (1982) determined dissolution half-times of 17 nickel compounds in water, rat serum, and renal cytosol (Table 5). The half-times for nickel compounds ranged from several days to years, and, in general, were less in biological systems than in water. Differences in rates of dissolution, however, cannot account for the striking differences in carcinogenic potency displayed by crystalline nickel subsulfide and amorphous nickel monosulfide, since both of these compounds have similar dissolution half-times in serum (34 and 24 days, respectively).

In contrast to particulate compounds, soluble nickel salts dissociate readily in the aqueous environment of biological membranes, facilitating their transport as metal ions

[Ni(II)]. Ni(II) has been demonstrated to cross cell membranes via Ca(II) channels and compete with Ca(II) for specific receptors (Brommundt et al., 1987; Kavaler et al., 1987). The intracellular nickel concentrations that are achieved by the Ca(II) transport route are probably lower than those reached by endocytosis, and this may explain the greater carcinogenic activities of certain particulate nickel compounds compared to soluble nickel salts. Increases in the concentration of nickel ions outside the cellular membrane have also been shown to inhibit phagocytosis, and may effect the ability of nickel to reach an intracellular target site (Costa and Heck, 1982,1983; Kuehn et al., 1982). Insoluble compounds may also increase cellular proliferation through the particle effect by causing irritation inside a cell.

Nickel interactions with nuclear membranes or nuclear matrix proteins may lower the effective concentration of nickel in nuclei. Phagocytized nickel particles, such as Ni_3S_2 and NiO, have been shown to have an affinity for certain membrane structures, including the polynuclear membranes, vacuole walls, and numerous lipid structures (Nieboer et al., 1986; Evans et al., 1982).

2.6.4) Excretion

The excretory routes in animals and humans depend in part on the physicochemical properties of the nickel compound and the mode of intake. Unabsorbed dietary nickel is eliminated in the feces, whereas absorbed nickel is excreted in urine. Given the relatively low level of gastrointestinal absorption in humans, nickel fecal levels roughly approximate daily dietary intake (US EPA, 1986). Urinary nickel levels in unexposed people vary from 2 to 4 ug/l (US EPA, 1986). All body secretions (saliva, tears, sweat, milk) appear to have the ability to remove nickel from the body, but they are minor routes of elimination.

Water solubility plays an important role in the clearance of nickel from the body. Soluble nickel salts, such as nickel sulfate and nickel chloride, are rapidly excreted in the urine after a few days and show little evidence for tissue accumulation (Clary, 1975). Insoluble nickel compounds, however, often take several months or years to be eliminated, and accumulation may occur in the lungs after inhalation (Wehner and Craig, 1972).

2.6.5) Evidence for a Role in Human Physiology

Although a role for nickel in human physiology has not been confirmed directly, the collective evidence strongly suggests that nickel is important to human health (Siegel, 1989). The evidence includes: (i) low concentrations of nickel is found in all human tissues and fluids that have been assessed by sensitive and reliable analytical techniques (the total amount of nickel in the adult body is 10 mg), (ii) nickel is an essential component of plant and bacteria enzymes--although no nickel-requiring enzymes are known in vertebrates yet, (iii) nickel deficiency in a number of species results in pathology including reduced hematopoiesis, depressed growth, and metabolic alterations, (iv) nickel concentrations in human body fluids seem to be tightly regulated by a number of mechanisms, including kidney reabsorption and binding to macromolecular proteins, such as albumin and alpha-2-macroglobulin.

2.7 Nickel Genotoxicity and Mutagenesis

The genotoxicity of Ni(II) and its various chemical forms has been assessed using a variety of biochemical and cellular approaches (Table 6). These approaches can be grouped into two general classes: (i) techniques which measure cellular or biochemical perturbations which have the potential to lead to mutations or which signal that DNA damage has occurred, and (ii) techniques which measure actual heritable changes in the DNA sequence (mutations) in living organisms.

Results from the first class of techniques (Section 2.7.1) include: interaction of Ni(II) with DNA in solution, interaction of Ni(II) with DNA and protein in chromatin, Ni(II)-catalyzed oxidative damage to DNA bases and chromatin, and Ni(II)-induced DNA strand breaks. The ability of Ni(II) to induce sister chromatid exchanges, to inhibit DNA replication, and to affect DNA repair are also discussed.

Results in the next part (Section 2.7.2) include the second class of techniques that measure heritable changes in the DNA sequence in various test organisms. These include results from gene locus mutation assays in prokaryotic, eukaryotic, mammalian, and human cells. In addition, Ni(II)-induced chromosomal aberrations observed *in vitro* and *in vivo* in animals, and *in vivo* in exposed human subjects are described. The

ability of Ni(II) to produce chromosomal fragmentation as measured as micronuclei induction is also reviewed.

Finally, results from Ni(II) cellular transformation studies are described (Section 2.7.3). Although transformation is not a direct measure of genotoxicity, the transformed phenotype is closely related to the process of cancer induction and likely includes genotoxicity--including alterations in DNA sequence and DNA expression.

2.7.1) Evidence of Nickel-DNA Interaction

2.7.1.1)) Interaction of Ni(II) with DNA in Solution

Ni(II) has been shown to bind weakly to DNA in solution (Eichhorn, 1962; Kasprzak et al., 1986). Scatchard analysis suggests that there are at least three distinct binding sites in the DNA molecule, including DNA phosphate oxygens and the N7 positions of adenine and guanine (Marzilli, 1981). Ni(II) appears to have a higher affinity for DNA phosphates than for nucleotide bases (Eichhorn and Shin, 1968).

Ni(II)-DNA binding is inhibited by essential divalent cations (magnesium and manganese) and several amino acids (histidine, glutamine, cysteine) in solution (Kasprzak et al., 1986). Kaspzak and colleagues suggested that divalent cation metal salts compete with Ni(II) for DNA binding sites. *In vivo* studies in rats support this hypothesis, indicating that magnesium and manganese may be associated with a reduction in Ni(II)-induced muscle sarcomas (Kasprzak et al., 1985).

2.7.1.2)) Interaction of Ni(II) with DNA and Protein in Chromatin

Several investigators have demonstrated that nickel binds to DNA and protein in chromatin (Ciccarelli and Wetterhahn, 1984; Hui and Sunderman, 1980). Chromatin is a highly-organized DNA complex in cells that is associated with histone and non-histone proteins.

In vitro and *in vivo* studies suggest that Ni(II) binds more readily to proteins than DNA. Harnett et al. (1982) used radiolabeled compounds to study the binding of Ni(II) to cellular macromolecules of Chinese hamster ovary cells. Treatment of cells with 10 ug/ml ^{63}NiS and $^{63}NiCl_2$ resulted in Ni(II) binding to protein 100 times greater than

binding to RNA or DNA. *In vivo* studies in rat kidney and liver indicate that the amount and distribution of Ni(II)-bound chromatin is dependent upon the protein:DNA mass ratio (Ciccarelli and Wetterhahn, 1984). The authors speculate that the different levels of non-histone proteins associated with chromatin in rat kidney and liver may be a factor in the amount of nickel which has access and binds to DNA. However, many investigators have not reported their DNA isolation procedure in detail, making difficult to evaluate their results. It is possible that a considerable level of protein was still associated with the DNA isolated in their studies.

Further analysis of Ni(II)-chromatin interactions indicate that Ni(II) has a high affinity for heterochromatic regions of the chromosome (Sen and Costa, 1985). Heterochromatin is highly condensed and nuclease-resistant. Preferential binding of Ni(II) for this region may be related to several factors, including: (i) heterochromatin is the first type of DNA encountered as Ni(II) enters the nucleus, (ii) heterochromatin has a higher protein/DNA ratio than euchromatin, and (iii) heterochromatin may have a greater number of reactive sites than euchromatin for Ni(II) because of its condensed state.

Noncovalent interactions between Ni(II) and proteins in heterochromatin may alter DNA structure and affect the cycle of chromosome decondensation required for replication (Eichhorn and Shin, 1968). Nickel is suspected to compete with magnesium which is an essential divalent cation that maintains heterochromatin in its condensed state. Research indicates that magnesium can antagonize the genotoxicity, cell transformation, and animal tumor induction by nickel compounds (Kasprzak et al., 1986; Conway et al., 1987).

2.7.1.3)) Ni(II)-catalyzed Oxidative Damage to DNA

Ni(II) may cause DNA damage by generating highly reactive oxygen species including the superoxide radical (O_2^-), the hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) (for review, see Sunderman, 1989; Kawanishi et al., 1990). Oxygen radicals are known to be genotoxic, and may cause several types of DNA damage including DNA strand breaks, DNA deletions, and chromosome aberrations (Hsie et al., 1986; Brawn and Fridovich, 1981).

Ni(II)-catalysis of oxidative DNA damage has been demonstrated in DNA bases and chromatin *in vitro* (Nackerdien et al., 1991; Kasprzak and Hernandez, 1989).

Investigators observed that guanosine is oxidized to 8-hydroxy-2'-deoxyguanosine (8-OH-dG) by the addition of Ni(II) and H_2O_2 (Datta et al., 1992). The 8-OH-dG lesion may promote the misincorporation of deoxynucleoside triphosphates by DNA polymerase and result in guanosine to thymidine transversions (Shibutani et al., 1991).

Ni(II) is also associated with increased oxidation of DNA bases *in vivo* (Kazpzak et al., 1992). Eleven products that are typical of hydroxyl radical derivatives of DNA bases were measured in pregnant, nickel acetate-dosed rats (i.p. injection, 90 or 180 umol/kg BW). Ni(II) exposure increased the content of all of these products in both renal and hepatic chromatin. Interestingly, the content of 8-OH-dG increased significantly in the kidney but remained unchanged in the liver, which the authors suggest may be related to the observed tissue specificity of Ni(II).

In vivo studies of $NiCl_2$-dosed rats demonstrate that oxygen free-radicals are implicated in the pathogenesis of acute nickel toxicity, as observed by enhanced lipid peroxidation in target tissues (Sunderman et al., 1985; Donskoy et al., 1986). Carcinogenesis may be initiated directly by hydroxyl radicals or via lipid peroxidation. However, lipid peroxidation and its degradation products may not be major contributors to DNA strand breakage, as observed by detection of DNA strand breakage several hours prior to lipid peroxidation (Stinson et al., 1992).

Evidence suggesting that oxidative damage is the primary mechanism of Ni(II)-induced DNA damage is equivocal. Kawanishi and associates report that several nickel compounds readily induced DNA single and double strand breaks in human HeLa cells, and observed a 1.5 fold increase of 8-OH-dG over background in nickel subsulfide and nickel sulfide treated cells (Kawanishi, unpublished). Because the increase in strand breaks was proportionately much higher than the increase seen in oxidized DNA bases, these results suggest that the two endpoints may not be related.

2.7.1.4)) Ni(II)-induced DNA Strand Breaks

Ni(II) has been observed to induce DNA strand breaks in *in vitro* and *in vivo* test systems (Ciccarelli et al., 1981; Costa, 1989). Strand breaks are scissions in the DNA backbone that may occur when nucleotide subunits are modified or damaged or by breakage of the phosphodiester bonds.

In studies with mammalian cells *in vitro*, both water-soluble and water-insoluble nickel compounds induced single strand breaks (SSBs) determined by alkaline sucrose gradients (Robison et al., 1984) and alkaline elution (Patierno and Costa, 1985). Robison and Costa showed that $NiCl_2$ and crystalline NiS (1 ug/ml) induced DNA strand breaks in cultured CHO cells. Exposure to $NiCl_2$ for only 2 hours indicated a high degree of DNA strand breakage, while exposure to NiS for 24 hours produced breakage. Patierno and Costa also reported that crystalline NiS and $NiCl_2$ induced SSBs in CHO cells, and add that SSBs were repaired relatively quickly and occurred at both non-cytotoxic and cytotoxic nickel concentrations.

Single strand breaks have also been studied by alkaline elution *in vivo* (Ciccarelli et al., 1981). Rat kidney, liver, lung, and thymus nuclei were examined 3 and 20 hours after a single i.p. injection of nickel carbonate (15 or 20 mg/kg). Nickel carbonate was observed to induce SSBs in kidney and lung tissue but not the liver or thymus.

2.7.1.5)) Ni(II)-induced Sister Chromatid Exchanges

Ni(II) has also been demonstrated to induce sister chromatid exchanges (SCEs) in mammalian and human cells (Newman et al., 1982; Sen and Costa, 1986). The induction of SCEs suggests that DNA damage has occurred; however, the relationship between an increase in SCEs and mutagenesis is unclear. SCEs are usually observed at doses below those that cause chromosomal aberrations.

Sen and Costa (1986) showed that CHO cells treated with either $NiCl_2$ (100 uM) or crystalline NiS (10 ug/ml) exhibited a two-fold increase in SCEs compared with untreated cells (Sen and Costa, 1986). The investigators also observed that nickel-dosed cells showed preferential induction of SCEs in heterochromatic regions (long arm of the X chromosome) of the DNA. Similarly, in studies with cultured human lymphocytes treated with $NiCl_2$ (10^{-6} to 10^{-3} M), Newman et al. (1982) reported a two-fold increase in SCEs over controls. These increases and those observed in other SCE studies (Deng et al., 1988; Sen and Costa, 1985) suggest that N(II) is a relatively weak inducer of SCEs.

2.7.1.6)) Ni(II) Interference with DNA Replication and Repair

Ni(II) has been demonstrated to interfere with the fidelity and kinetics of DNA replication and repair (for review, see Coogan et al., 1989; Sirover and Loeb, 1976). Ni(II) may interact directly with the DNA substrate or with the enzymes that are responsible for DNA synthesis and excision repair.

DNA repair of strand breaks is induced by several carcinogenic nickel compounds in mammalian test systems (Sunderman, 1985, 1989). Robison et al. (1983, 1984) showed that cells exposed to $NiCl_2$, Ni_3S_2, and crystalline NiS exhibited enhanced DNA repair activity *in vitro*. Hamilton-Koch et al. (1985) used a nick-translation assay to detect DNA damage in cultured human fibroblasts exposed to a subcytotoxic concentration of $NiCl_2$; they reported that DNA strand breaks induced by Ni(II) were repaired within 2 hours after exposure.

Other studies suggest that Ni(II)-exposed cells lose their ability to repair damaged DNA. Swierenga and colleagues (1968) examined DNA repair in rat hepatocytes by measuring unscheduled DNA synthesis audioradiographically after exposures to $NiCl_2$ and Ni_3S_2. They observed the inhibition of excision repair of DNA damage induced by methylmethanesulfonate, a potent alkylating agent. Similarly, Hatwig and Beyersmann (1988) showed that $NiCl_2$ inhibits the excision repair of mutations induced at the *hprt* locus in V79 CHO cells by exposure to ultraviolet light.

Several investigators have also examined Ni(II) interactions with the fidelity of DNA replication (for review, see Coogan et al., 1989; Sirover and Loeb, 1976). Ni(II) in the presence of Mg(II) (5 to 10 mM) decreased polymerase activity significantly and increased misincorporation (Miyaki et al., 1977). Ni(II) is suspected to compete with Mg(II), an essential divalent cation that helps regulate replication fidelity.

2.7.2) Evidence of Heritable Changes in DNA

2.7.2.1)) Gene Locus Mutation Assays

Gene locus assays have been widely used to study the mutagenicity of nickel in prokaryotes, eukaryotes, mammalian cells, and human cells (for review, see Heck and

Costa, 1983). These assays indicate actual heritable changes in the DNA of living organisms.

The majority of reversion and forward assays including Salmonella *his*$^-$, Bacillus subtilis *rec*$^+$, and Escherichia coli *trp*$^-$ suggest that Ni(II) is not mutagenic in bacteria. Weakly positive results, however, were obtained for $NiCl_2$ at various concentrations in a simplified fluctuation test utilizing a homoserine-dependent strain of Corynebacterium (Pikalek and Necasek, 1983). Several investigators have suggested that the negative results obtained in reversion assays may be related to the lack of phagocytic activity in bacteria; however, Biggart and Costa (1986) reported negative results in the Ames assay under conditions which resulted in a substantial uptake of $NiCl_2$. The negative results in reversion assays may be attributed to the fact that this type of assay is limited in the detection of certain types of mutations associated with Ni(II)-induced damage, specifically large DNA alterations.

Singh (1983) reported Ni(II)-induced gene conversion and reverse mutations in the yeast *Saccharomyces cerevisiae*. These organisms were exposed to nickel sulfate (0.1M). Few mutation assays have been conducted in lower eukaryotes; however, they are capable of measuring several mutation endpoints including deletions, gene conversion, and chromosome loss.

Experiments with mammalian cells have shown that nickel may be weakly mutagenic, especially in forward mutation assays determined at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) or thymidine kinase (*tk*) loci. In nickel chloride-treated Chinese hamster V79 cells, a two to three-fold increase was observed over background (Miyaki et al., 1979). Negative results were obtained at the same locus under similar conditions (Nishimura and Umeda, 1978). A subsequent study in L5178Y/$tk^{+/-}$ mouse lymphoma cells revealed that $NiCl_2$ produced a small increase in $tk^{-/-}$ mutations monitored by trifluorothymidine resistance (Amacher and Paillet, 1980). Several other investigators have observed weak mutagenic responses at the *hprt* locus (Costa et al., 1980b; Swierenga and McLean, 1985).

Large variations in the mutagenic responses observed for Ni(II)-treated cultured cells may be due in part to the toxicity of nickel compounds, and in particular, the ability of Ni(II) to block replication and prevent the expression of mutation. While sufficient time is required for nickel to enter cells and reach targets in the nucleus, prolonged

treatments of several days often produce large reductions in cell viability. In addition, species differences may cause variations between test systems with respect to Ni(II)-induced toxicity.

2.7.2.2)) Ni(II)-induced Chromosomal Aberrations

Chromosomal aberrations induced by Ni(II) have been studied by several investigators *in vitro* and *in vivo* (for review, see US EPA, 1986). They include many different types of clastogenic DNA damage, such as, chromosome breaks, gaps, deletions, and rearrangements.

In vitro studies of Ni(II)-induced chromosome aberrations have been conducted in mammalian and human cell systems (Nishimura and Umeda, 1979; Christie et al, 1988). Nishimura and Umeda (1979) exposed FM3A mammary carcinoma cells derived from C3H mice to various concentrations (1.0×10^{-3} to 3.2×10^{-4} M) of nickel chloride, nickel acetate, and nickel subsulfide for 24 and 48 hours. Nickel chloride and nickel acetate induced no aberrations, while nickel subsulfide at the same concentrations induced 6 to 14-fold increases over the control value (2%) after 48 hours of treatment. Larramendy et al. (1981) investigated the clastogenic effect of nickel sulfate in human lymphocyte cultures. Nickel sulfate (1.9×10^{-5}) induced 14 aberrations in 125 metaphase cells (11.2%) following a 48 hour treatment; whereas the background frequency was three aberrations in 200 metaphases or (1.5%).

Further research in experimental animals and nickel-exposed workers also suggests that Ni(II) causes chromosome aberrations *in vivo*. Sunderman et al. (1990) administered nickel subsulfide to male Fischer 344 rats by i.r. injection (20 mg/rat) and examined chromosomal aberrations in renal tissue. After 21 months, malignant neoplasms were reported in 6 out of 28 rats (compared to 0 out of 13 for controls). The authors report prominent chromosomal aberrations in three of the neoplasms. In humans increases in chromosome aberrations have been observed in the peripheral lymphocytes of nickel exposed workers (Deng et al, 1988; Senft et al., 1992). Senft et al. (1992) reported that nickel sulfate production workers exhibited a slight increase in aberrations (5.2) above controls (4.0); whereas nickel oxide exposed individuals had a more significant increase (9.5).

Collectively, the evidence demonstrates that Ni(II) induces chromosome aberrations in mammalian and human cells *in vitro* and *in vivo*, although the level of effects vary in different test systems. This may be related to the fact that uptake and availability of Ni(II) was not fully considered in several study designs. The timing of the assay and recovery in metal-free medium appears to be crucial--presumably because metal inhibits the replication necessary to observe the effects.

2.7.2.3)) Chromosomal Fragmentation and Micronuclei Induction

Chromosomal fragmentation and micronuclei induction have been also observed in Ni(II)-treated mammalian and human cells *in vitro* (Sobti and Gill, 1989; Montaldi et al., 1987). Micronuclei are broken chromosomes that are encapsulated in small nucleus-like structures in the cell. Micronuclei induction is considered to represent an actual mutagenic event since it results from chromosomal fragmentation.

Administration of $NiNO_3$, $NiCl_2$, and $NiSO_4$ (72.2, 95, 73 mg/kg, respectively) to mice produced statistically significant increases in the frequency of micronuclei in the polychromatic erythrocytes in bone marrow (Sobti and Gill, 1989). Increases in the frequency of spermatozoa with abnormal heads was also observed. Similarly, increases were observed in micronucleated bone marrow cells of albino Swiss mice treated with an i.p. injection of NiF_2 (Bhunya and Pal, 1986), and in $NiCO_3$-treated (10-6 to 10-3 M) human lymphocytes *in vitro* (Montaldi et al., 1987).

2.7.3) Ni(II)-Induced Cellular Transformation

Both soluble and particulate nickel compounds promote morphological cell transformation *in vitro* (for review, see Sunderman 1984). Costa et al. (1989) and DiPaolo and Casto (1979) reported that exposure of SHE cells to nickel subsulfide or nickel sulfate produce high incidences of transformed foci *in vitro*, whereas exposure to amorphous nickel sulfide, which is noncarcinogenic in animal bioassays, yields negative results.

Hansen and Stern (1984) examined nickel-induced transformation and toxicity of baby hamster kidney cells (BHK-21) by nickel compounds by growth in soft agar and 5-day growth in culture, respectively. Five compounds were assayed: (i) three particulates--

NiO, Ni_2O_3*$6H_2O$, and Ni_3S_2, (ii) one soluble compound--$Ni(CH_3COO)_2$, (iii) one aerosol--Ni welding fumes. Although the nickel compounds varied substantially in their transforming potencies, the compounds produced the same proportions of transformed colonies when tested at equitoxic dosages (e.g. 50% survival). The authors conclude that the intracellular bioavailability of Ni(II) is the property that determines the transforming potency of nickel compounds.

2.8 Nickel Carcinogenesis

2.8.1) Epidemiological Studies

Epidemiological studies have shown that exposure to certain nickel compounds is associated with human cancer in nickel refinery workers. However, much remains unknown about the mechanisms by which nickel induces carcinogenesis. Exposure to nickel sulfides, nickel oxides, and soluble nickel showed increased incidence of tumors in the lung and nasal passages in nickel workers (Pedersen et al., 1973; Doll et al., 1977; Chovil et al., 1981). Excess risks for cancers of the larynx, kidney, and prostate have also been reported in nickel-exposed workers; however, they are significantly lower than those observed for respiratory tract cancer (Doll, 1983), and they may be confounded by several factors including exposure to multiple compounds (arsenic, chromium, cobalt, benzo[a]pyrene) in the workplace.

Changes in nickel processing procedures since 1925 have significantly reduced nickel exposure in the work environment. These changes include the use of personal protective equipment, such as masks and respirators, as well as improvements in ventilation, and the use of arsenic-free sulfuric acid in refinining. Since these improvements, excess risks for cancer in nickel refinery workers has sharply decreased.

2.8.2) Experimental Animal Studies

Nickel compounds are carcinogenic in a variety of species, including rats, mice, hamsters, rabbits, and guinea pigs (Sunderman, 1984; US EPA, 1986). The carcinogenic response varies significantly depending upon the nickel compound, the animal species and strain, and the route of exposure.

NiO, $Ni_2O_3 \cdot 6H_2O$, and Ni_3S_2, (ii) one soluble compound--$Ni(CH_3COO)_2$, (iii) one aerosol--Ni welding fumes. Although the nickel compounds varied substantially in their transforming potencies, the compounds produced the same proportions of transformed colonies when tested at equitoxic dosages (e.g. 50% survival). The authors conclude that the intracellular bioavailability of Ni(II) is the property that determines the transforming potency of nickel compounds.

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Intramuscular injection of particulate nickel compounds has been observed to induce tumors in animals, generally at the site of injection. Particularly potent are the crystalline nickel sulfides such as crystalline NiS and Ni_3S_2, which have been shown to produce typically a 90-100% tumor incidence at any site into which they are experimentally introduced (Jasmin and Riopelle,1976; Sunderman and Maenza, 1976). In a comparison of 18 different nickel compounds at equivalent doses (intramuscular injection of male Fischer rats, 14 mg Ni/rat), Sunderman placed nickel compounds into five categories: Class A compounds (Ni_3S_2, NiS, Ni_4FeS_4) induced sarcomas at the injection site in 100% of the rats; Class B compounds (NiO, NiS_2, Ni_3Se_2, NiAsS, Ni_5As_2) induced sarcomas in 85-93% of the rats; Class C compounds (Ni dust, Nisb, NiTe, NiSe, $Ni_{11}As_8$) induced sarcomas in 50-65% of the rats; Class D compounds (amorphous NiS, $NiCrO_4$) induced sarcomas in 6-12% of the rats; and Class E compounds (NiAs, $NiTiO_3$, NiFe alloy) along with control vehicle injections induced no sarcomas. These results suggest that nickel compounds exhibit extremely different carcinogenic potencies *in vivo* (Sunderman, 1984, 1985).

In contrast to particulate nickel compounds, soluble nickel salts [$NiCl_2$, $NiSO_4$, and $Ni(CH3OO)_2$] are not considered tumorigenic in animals by ingestion or injection, probably due to their rapid clearance from the body (Sunderman and Maenza, 1976). Daily injections of $NiCl_2$, however, (500 or 750 umol/kg, s.c. in male Fisher rats) were found to cause severe lung damage including hyperplasia, cellular atypia, and frequent mitoses (Coogan et al., 1989). Although these lesions were the result of subcutaneous exposure, they are similar to those produced by direct inhalation. In another study, repeated intraperitoneal injection (93 times a week for 8 weeks in mice) of nickel acetate gave a 3-fold increase in lung tumors compared to controls (Sunderman, 1981). This evidence suggests that nickel mimics other known lung carcinogens that also target the lungs after treatment by different exposure routes (Sunderman, 1988).

Inhalation animal studies also suggest that nickel compounds are carcinogenic *in vivo*, and may be more relevant to the assessment of potential human risk than intraperitoneal studies (US EPA, 1986). In one study, Fischer 344 rats were exposed to Ni_3S_2 (0.97 mg Ni/m^3) by inhalation resulting in hyperplasia, metaplasia, adenomas, and adenocarcinomas equally in both males and females (Ottolenghi et al., 1974). The authors observed the preneoplastic lesions and tumors in both bronchiolar and alveolar regions of the lung. Although several other inhalation studies of nickel have been

conducted in hamsters, this species may not be very sensitive to carcinogens by inhalation.

Nickel has also been demonstrated to be carcinogenic in animals that are generally resistant to chemical carcinogenesis, such as the Japanese common newt (*Cynops pyrrhogaster*) (Okamoto, 1987). Intraocular injection of a single dose (100 ug) of Ni_3S_2 was invariably turmorigenic in these animals, inducing malignant melanoma tumors in seven out of eight animals. No other carcinogen is known to produce tumors in this organism (Coogan et al., 1989).

In summary, nickel compounds are carcinogenic in many animal species and by different routes of administration. Several inhalation studies have been poorly designed or used insensitive models (e.g. hamsters), making it difficult to evaluate carcinogenic effects *in vivo*. In general, Ni_3S_2, $NiSO_4$, and $Ni(CO)_4$ are tumorigenic by inhalation exposure in rats and mice--with Ni_3S_2 being the most potent.

2.9 Summary of Ni(II)-Induced Genotoxicity, Mutagenicity, and Carcinogenesis

Ni(II) exhibits both genotoxic and carcinogenic effects in humans, animals, and *in vitro* test systems. Epidemiological studies of nickel-exposed workers show increased risks for respiratory cancer, and experimental animal studies show increases in tumor incidence by several routes of exposure. Ni(II) has also been shown to bind to protein and DNA in chromatin, and induce multiple types of DNA damage--including strand breaks, sister chromatid exchanges, and DNA-protein crosslinks. Ni(II) also causes heritable changes in the DNA of mammalian and human cells *in vitro* and *in vivo*. These changes have been observed by increases in mutation frequency in gene locus assays, and the induction of micronuclei and chromosome aberrations. And finally, Ni(II) has induced cellular transformation in several *in vitro* assays, which are predictive of carcinogenicity.

3. Materials and Methods

3.1 Chemicals and Media Components

The nickel compounds were obtained from the following sources: nickel sulfate heptahydrate and nickel hydroxide--Aldrich Chemical Company (Milwaukee, WI); nickel oxide (green)--Inhalation Toxicology Institute (Albuquerque, NM); nickel subsulfide--INCO, LTD (Ontario, Canada); nickel metal powder--Sheritt Gordon, LTD (Alberta, Canada). Nickel subsulfide was stored under an argon atmosphere, and all five compounds were kept in containers that prevented light exposure. Trifluorothymidine (TFT), 6-thioguanine (6-TG), +-alpha-tocopherol succinate (vitamin E), aminopterin, cytidine, hypoxanthine, and thymidine were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (H_2O_2) was purchased from Mallinckrodt Specialty Chemical Co. (Paris, KY).

Media components were obtained from the following sources: equine serum (Hyclone Laboratories, Logan, UT); RPMI, HEPES buffer, and 1000X pen\strep (Lineberger Cancer Research Center, Tissue Culture Facility, UNC-CH).

3.2 Cell Culture

The origin and characterization of TK6 human lymphoblastoid cell line has been previously described (Skopek et al., 1978; Liber and Thilly, 1982). Stock TK6 cells were maintained in RPMI 1640 + L-glutamine supplemented with 10% heat-inactivated equine serum, 2% HEPES buffer, and 1X pen/strep. Cells were grown in 75 cm^2 tissue-culture flasks in a humidified incubator (6% CO_2) at 37°C and diluted daily to 3 X 10^5 cells/ml. For heat inactivation, the serum was placed in a water bath for 1 hour at 56°C. Prior to mutation experiments, TK6 cells were treated to reduce spontaneous *hprt*$^-$ and *tk*$^{-/-}$ mutants with cytidine, hypoxanthine, aminopterin, and thymidine (CHAT) for 2 days, followed by 1-2 day(s) in medium containing only cytidine, hypoxanthine, and thymidine (Liber and Thilly, 1982).

3.3 Exposure Conditions

All nickel stock solutions were prepared immediately prior to dosing. Nickel sulfate was dissolved in sterile deionized ultra-filtered water (Fisher Scientific, Fair Lawn, NJ). Nickel subsulfide, nickel hydroxide, nickel oxide, and nickel metal powder were insoluble in any biologically-compatible solvent, and therefore delivered as a suspension. Stock solutions for these compounds were made with complete medium and placed in an ultrasound water bath (Fisher Scientific, Fair Lawn, NJ) for 10 minutes. Prior to each aliquot, the nickel solutions were shaken vigorously to obtain a uniform mixture for dosing.

The mutation assay exposure summary is outlined in Figure 1. For each experimental point, 200 mls of cell suspension at 3 X 10^5 cells/ml (6 X 10^7 cells total) were treated in 225 cm^2 tissue culture flasks to ensure the survival of a statistically significant number of mutants. NiO treated cultures contained only 1.5 X 10^7 cells total. TK6 cells were exposed to nickel compounds for 8 hours to permit uptake, and then exposed for an additional 16 hours following the addition of 20 uM H_2O_2. Nickel concentrations were chosen to yield greater than 10% survival.

Following exposure, nickel sulfate treated cultures were pelleted by centrifugation (1000 rpms, 4 min), and resuspended at 3 X 10^5 cells/ml in 200 mls of complete medium. For the insoluble nickel compounds, dosing and exposure conditions were similar to those for nickel sulfate experiments, however, the compounds could not be completely removed by centrifugation because they were insoluble in the culture media over the 24 hour period. For these compounds, the nickel was removed by diluting the cell cultures daily to 3 X 10^5 cells/ml with fresh culture media. This reduced the nickel concentration by 50-67% each day. Culture growth was monitored daily with a Coulter Counter (Coulter Diagnostics, Hialeah, FL).

3.4 Toxicity and Mutant Fraction Determination

For toxicity determination, treated cells were tested for their colony-forming ability by diluting and plating 2 cells/well in multwell microtiter plates (4 plates/culture) immediately after exposure. The plates were incubated at 37°C in a humidified incubator (6% CO_2) and scored after 14 days. The average number of colony-forming units/well is calculated utilizing Poisson statistics and is equal to -ln [(negative

wells)/(total wells)]. Relative survival (% survival) is then calculated by dividing the colony-forming unit/well of treated cells by that of the zero control and multiplying by 100%. Survival determined by toxicity plating at 2 cells/well may not reflect survival in bulk cultures. This is because the nickel concentration in the plates measuring toxicity is several orders of magnitude less than the nickel concentration in the bulk culture (due to the dilution of the culture media from 3 - 10 X 10^5 cells/ml to 10 cells/ml for toxicity plating). However, monitoring the growth of the cultures after nickel exposure can be used as an additional assessment of toxicity by extrapolating the exponential portion of the growth curve back to the treatment time (DeLuca et al., 1983).

For mutant fraction determination, treated cells were maintained at 3 X10^5 cells/ml by daily dilution to allow full expression of the mutant phenotype. Cultures were plated when the slowest growing culture had doubled approximately four times for the *tk*$^{-/-}$ mutant phenotype and nine times for the *hprt*$^-$ mutant phenotype. The mutant fraction was determined by plating 4 X 10^4 cells/well (4 plates/culture) with 1 ug/ml of trifluorothymidine (TFT) to select for *tk*$^{-/-}$ mutants and 1 ug/ml of 6-thioguanine (G-TG) to select for *hprt*$^-$ mutants. Colony forming efficiency was determined by plating in 96 well microtiter plates (Costar, Cambridge, MA) at 2 cells/well (2 plates/culture).

Because high concentrations of nickel oxide (16-50 mM) were used in mutation experiments, nickel oxide was not easily removed from cultures by daily dilution, and residual compound was observed in *tk* mutant fraction determination plates. To reduce the possibility of growth inhibition caused by nickel oxide in the selection plate wells, nickel oxide treated cultures were maintained for 1-2 days longer prior to plating at the *tk* locus to remove additional nickel oxide by the daily dilution of the cell cultures. The TK6 cells were also plated at 2 X 10^4 cells/well (8 plates/dose) during *tk* mutagenicity plating to further reduce the nickel concentration in the microtiter plates.

Two phenotypic classes of TFT mutants can arise during selection with TFT (Yandell et al., 1986). One class of mutants has a normal growth rate relative to wild-type TK6 (normal-growing *tk*$^{-/-}$), while the second class of mutants grows at a slower rate (slow-growing *tk*$^{-/-}$). To assess the frequency of slow-growing *tk*$^{-/-}$ mutants, the microtiter plates used to determine the TFT mutant fraction were incubated in a humidified 37^{o}C incubator (6% CO_2) for 10 days, scored for normal-growing *tk*$^{-/-}$ mutants and then re-fed on day 10 with 25 ul/well of complete medium containing 10 ug/ml of TFT (final concentration in the microtiter wells was approximately 1 ug/ml). The plates were

reincubated for 10 days, and the total number of positive wells were scored using a dissecting microscope. The number of slow-growing $tk^{-/-}$ mutants was the difference between the number of positive wells scored between day 10 and day 20. Plates containing 6-TG were scored for *hprt*⁻ mutants 14 days after plating.

3.5 Radical Scavenger Experiments

Radical scavenger experiments were performed with vitamin E (25 uM) (+-alpha-tocopherol acid succinate). Dosing and plating conditions to determine slow-growing $tk^{-/-}$ were identical to those described above. Cultures were treated with a single nickel dose that yielded a positive mutagenic response for slow-growing $tk^{-/-}$ mutants in the absence of vitamin E [nickel sulfate (175 uM), nickel subsulfide (250 uM), nickel hydroxide (600 uM), and nickel metal powder (1000 uM)]. Immediately following nickel treatment, cells were dosed with vitamin E (25 uM) dissolved in DMSO. This concentration of vitamin E was selected because it was as high as possible without causing cytotoxicity to TK6 cells.

Vitamin E has the ability to act as a free-radical scavenger and radio-protective agent, presumably due to its antioxidant properties (Dean and Cheesman, 1987; Lieber et al., 1986). Vitamin E has also been shown to protect against the carcinogenic and or mutagenic activity of ionizing radiation and chemical agents (Ames, 1983; Borek et al., 1986; Gebhart et al., 1985; Kalinina et al., 1979; Radner and Kennedy, 1986) and to inhibit the formation of chromosomal aberrations induced by crystalline NiS particles (Lin et al., 1991).

3.6 Format for Mutation/Toxicity Data Reporting

A uniform format was created for reporting mutation/toxicity results. This format captures all of the raw data produced by a mutation experiment and also contains calculated values based on the raw data in individual data tables for each experiment. Since these tables are used to report most of the data collected in the study, a description of the data entries is given here.

3.6.1) Table Title

The title indicates the compound under study and the experiment number for cross-referencing to the laboratory notebook.

3.6.2) Main Table Headings

Test Compound (uM/mM)

This gives the concentration of nickel compound used in the experiment. Numbers are based on the amount of compound added to the culture and not the amount of soluble Ni(II) actually in solution. The notation "+ H_2O_2" indicates the inclusion of 20 uM H_2O_2 during exposure. Note that the range of nickel compound concentration tested with and without H_2O_2 was sometimes different in order to compensate for the toxic effects of H_2O_2.

Toxicity

The toxicity of the treatment to the cells was measured immediately after exposure. To this end, cells were tested for their colony forming ability by diluting and plating 2 cells/well in multiwell microtiter dishes. The total number of wells scored (T wells) and the number of empty wells (-wells) are given in the table. The average number of colony forming units/well is calculated utilizing Poisson statistics and is equal to -ln [(-wells)/(Total wells)]. Relative survival (% Survival) is then calculated by dividing the colony forming units/well of treated cells by that of the zero control and multiplying by 100%.

Surviving Mutants

These numbers are calculated values and are included to evaluate the significance of mutation results. They represent an estimate of the number of viable mutants contained in the cultures immediately after treatment. The number of surviving mutants is given for each mutation endpoint measured, including normal-growing *tk*$^{-/-}$ mutants, slow-growing *tk*$^{-/-}$ mutants and *hprt*$^{-}$ mutants. The values given for each treated culture is equal to: (% Survival)(number of cells treated)(observed mutation frequency). The

number of cells treated is equivalent to 6×10^7 unless otherwise specified.

tk-Normal, tk-Slow, and hprt

These entries give the plate counts and calculated mutation frequencies for each mutation endpoint. For each mutation endpoint "+ wells" gives the total number of positive wells (mutant colonies) observed on plates containing the selective agent [trifluorothymidine (*tk*) or 6-thioguanine (*hprt*)] and "T wells" gives the total number of wells with selective agent that was scored. Approximately 40,000 cells per well were seeded for mutant selection. The plating efficiency of the cultures (PE (%)) was determined at the time of selection by plating 2 cells/well and observing the number of negative wells (PE-wells) among the total number of plating efficiency wells (PE T wells). PE (%) is equal to -ln[(PE-wells)/(PE T wells)][100%] /2. Mutation frequency (MF) is equal to -ln{[(T wells)-(+wells)]/(T wells)}/(40,000)(PE)(0.01). The determination of normal-growing $tk^{-/-}$ and slow-growing $tk^{-/-}$ mutants are accomplished with the same set of plates (plates are scored at different times for normal- and slow-growing $tk^{-/-}$ determination) and consequently the plating efficiency data are only reported once in the *tk*-Normal section of the table.

3.7 Statistical Analysis

Statistical analysis of observed mutant fractions was performed using the maximum likelihood method. The underlying assumption in the analysis was that the number of mutants per well followed a Poisson distribution. Evaluation of the equivalence of mutant fractions in control and treated groups was based on standard normal z-tests utilizing estimated variances in observed mutant fractions. All p-values reflect two-sided testing. Since p-values were calculated for each nickel treatment and there were several treatments at a given nickel concentration, p-values at each dose point were added together and then divided by the square root of the number of observations. Statistical signficance was determined by a p-value less than 0.05.

4. Results

4.1 Mutagenic Potential of Nickel Compounds in Human Cells *In vitro*

The mutagenic potential of nickel sulfate, nickel subsulfide, nickel hydroxide, nickel oxide, and nickel metal powder was determined using a minimum of three independent experiments for each compound (see table below). Tables 7-24 contain all the raw data collected for mutation experiments [table format and contents were discussed previously (Section 3.6)]. Figures 2-10 summarize mutation results, and depict the induced mutant fraction (observed mutant fraction minus the mutant fraction in the concurrent control culture) as a function of dose.

Mutation Experiment Summary

Compounds	*hprt*$^-$	*tk*$^{-/-}$ Normal	*tk*$^{-/-}$ Slow	Tables	Figures
Nickel Sulfate	-	-	+	7-10	2-3
Nickel Subsulfide	-	-	+	11-15	4-5
Nickel Hydroxide	-	-	+	16-18	6-7
Nickel Metal Powder	-	-	+	19-21	8-9
Nickel Oxide	-	-	-	22-25	10

The mutant frequency for controls averaged across experiments for *hprt*$^-$, normal-growing *tk*$^{-/-}$,and slow-growing *tk*$^{-/-}$ mutants were 2.75 ± 1.32, 1.54 ± 1.02, and 3.83 x 10^{-6} ± 1.13, respectively. No dose-related increase was observed above background in the frequency of *hprt*$^-$ mutants or normal-growing *tk*$^{-/-}$ mutants for the tested compounds. These results indicate that the nickel compounds do not efficiently induce base pair substitutions, frameshift mutations, or small deletions in human cells. However, nickel sulfate, nickel subsulfide, nickel hydroxide and nickel metal powder induced slow-growing *tk*$^{-/-}$ mutants. This phenotype has been previously shown to be associated with large DNA alterations involving the *tk* gene (Yandell et al, 1986; 1990). Significant induction of slow-growing *tk*$^{-/-}$ mutants occurred only at nickel concentrations that were cytotoxic (Table 31). No significant increase in the frequency of slow-growing *tk*$^{-/-}$ mutants was observed with nickel oxide.

4.2 Mutagenic Potential of Nickel Compounds In the Presence of Hydrogen Peroxide and In the Presence of Vitamin E

To investigate the effect of added hydrogen peroxide on the mutagenicity of these nickel compounds, nickel-treated cells were exposed to H_2O_2 (20 uM). This concentration of H_2O_2 alone resulted in measurable toxicity and mutagenicity averaged across experiments [survival: 77%; induced mutant frequency for *hprt*$^-$: 0.75 X 10^{-6}; normal-growing *tk*$^{-/-}$: 0.68 X 10^{-6}; slow-growing *tk*$^{-/-}$ mutants: 1.65 X 10^{-6}]. These increases were only significant for slow-growing *tk*$^{-/-}$ mutants ($p = 0.0033$, Mann-Whitney, unpaired, two-tailed test).

The addition of H_2O_2 to nickel-treated cultures shifted the dose-response relationship for slow-growing *tk*$^{-/-}$ mutants to lower nickel concentrations, but the effect was less than a factor of two (Figures 2-10). The shape of the curve for slow-growth *tk*$^{-/-}$ mutants was similar for nickel-treated cultures in the absence and presence of H_2O_2.

To further explore the possible role of reactive oxygen species in nickel mutagenesis, cells were treated with nickel in the absence and presence of the radical scavenger vitamin E (25 uM). A single dose was selected for each of the four nickel compounds that yielded a positive mutagenic response for slow-growing *tk*$^{-/-}$ mutants in the mutation experiments discussed previously. Results from three radical scavenger experiments suggest no significant decrease in the observed mutation frequency in the presence of vitamin E [Table 25-27; Table 28 (summary)].

4.3 Reconstruction/Validation

Because nickel-treated cultures were dosed at several different nickel concentrations in a given experiment, TK6 cells exhibited marked variation in their growth rate from the cytotoxic effects of nickel. To ensure phenotypic expression in all cultures, plating in selective media was performed when the slowest growing culture had doubled approximately four times for the *tk*$^{-/-}$ mutant phenotype and nine times for the *hprt*$^-$ mutant phenotype. As a result, TK6 cells exposed to lower nickel concentrations often

achieved several doublings beyond that required for phenotypic expression before being plated. TK6 cells dosed at highly cytotoxic nickel doses grew more slowly, and therefore, were plated immediately after the number of required doublings for expression of the phenotype. Therefore, it is possible that in the cultures treated with lower nickel concentrations, slow-growing $tk^{-/-}$ mutants could express the slow-growth phenotype and be diluted out due to their slower growth rate, thus artificially depressing their apparent frequency in the cultures. This would tend to extenuate the nonlinear nature of the dose-response curve for slow-growing $tk^{-/-}$ mutants.

To confirm that plating after different doubling times for $tk^{-/-}$ mutants did not bias our results, TK6 cells were treated at a low concentration of nickel sulfate (60 uM) and plated after four and nine doublings in the presence of the selective agent TFT. Soluble nickel sulfate was chosen because treatments could be terminated by precipitating the cells by centrifugation and resuspending cells in fresh media. This concentration of nickel sulfate produced measurable toxicity and mutagenicity in previous mutation experiments. Eight 200 ml cultures were maintained for the experiment (four nickel sulfate-dosed cultures, four respective controls). Exposure and plating conditions were the same as noted for previous mutation experiments.

No significant difference was observed for normal-growing and slow-growing $tk^{-/-}$ mutants plated after four and nine doublings (Mann-Whitney, unpaired, two-tailed test, p value 0.8857 and 0.3429, respectively (Table 29). We conclude that plating after the required number of doublings for phenotypic expression did not bias our results.

An additional experiment was conducted to confirm the phenotype of slow-growing $tk^{-/-}$ colonies quantitated in our study. TK6 colonies that were scored positive as slow-growth mutants in mutation experiments were picked from microtiter plates and monitored for cell growth. Cells were maintained in complete medium (10 mls) and TFT (1 ug/ml). Compared to normal-growing TK6 cells (doubling time 16-19 hrs), the cells grew more slowly (doubling 24-36 hrs) which is characteristic of the slow-growing $tk^{-/-}$ phenotype. This experiment confirmed that the colonies scored as slow-growing $tk^{-/-}$ mutants were actually the slow-growth phenotype.

5. Discussion

5.1 Mutagenic Potential of Nickel Compounds *in vitro*

Nickel compounds have been demonstrated to induce mutation and several other types of DNA damage in mammalian cell systems *in vitro* (for review, see Coogan et al., 1989; US EPA, 1986). In the present study, the induction of mutation by certain nickel compounds was investigated in cultured human TK6 lymphoblasts.

The TK6 cell line has been previously used to determine the mutagenic potential of several chemical and physical carcinogens (Liber and Thilly, 1982; Kodama et al., 1989; Liber et al, 1989). The TK6 line is particularly useful because it allows direct comparisons of mutation at the *hprt* and *tk* gene loci. Both *hprt* and *tk* are expected to respond to point mutations, frameshifts, and deletions up to several base pairs. In addition, the *tk* gene has been demonstrated to be sensitive to large DNA alterations in the *tk* region in both human and mouse cells (Yandell et al., 1986; Moore et al., 1988).

Two different types of *tk* mutant phenotypes have been identified that are characterized by different growth rates and different types of mutation (Yandell et al., 1986, 1989). Normal-growing $tk^{-/-}$ mutants have a doubling time similar to wild-type $tk^{+/-}$ cells (16-19 hrs) while slow-growing $tk^{-/-}$ mutants double between 24-38 hours. Southern blot analysis shows that the normal-growth mutants contain the active *tk* allele, suggesting that the mutations produced in this phenotype are point mutations, frameshifts, and deletions. Similar analysis of slow-growth mutants demonstrates that these mutants have lost the entire active *tk* allele, and suggests the induction of large DNA alterations. Comparisons of the mutation frequency for these two *tk* mutant phenotypes can provide some critical information about the type(s) of DNA damage that is induced by nickel compounds.

In the present study, nickel compounds were demonstrated to increase the frequency of slow-growing $tk^{-/-}$ mutants, but failed to induce normal-growing $tk^{-/-}$ and $hprt^{-}$ mutants. These results suggest that certain nickel compounds induce large DNA alterations and not point mutations, frameshifts, or small deletions in human cells.

These DNA alterations may include large deletions, gene conversion, and reciprocal recombination.

Analysis by genetic markers show that the induction of the slow-growth phenotype often involves the loss of the active *tk* allele, assumed also to remove flanking essential genes (Yandell et al, 1990). Since the *tk* locus is on an autosome, these events are not cytotoxic, and these mutants can be recovered and quantified. The slow-growing phenotype is speculated to be caused by the deletion of one or more flanking essential genes. Analogous findings have been reported in L5178/$tk^{+/-}$ mouse lymphoma cells (Moore et al., 1988).

At the hprt locus, TK6 cells are hemizygous and a large deletion or DNA alteration that includes the loss of essential flanking genes would be lethal (Christie et al., 1992). These mutants are not recoverable, and therefore, are not quantified in the TK6 system. The failure of nickel compounds to induce *hprt*$^-$ mutants in this study, however, confirms the negative results obtained for normal-growing $tk^{-/-}$ mutants.

Two possible explanations are discussed for the apparent nonlinear response of the slow-growth phenotype in our study. Because nickel-treated cultures were dosed at several different nickel concentrations in a given experiment which resulted in marked variation in growth rates, TK6 cells were plated at different doubling times to ensure phenotypic expression. Slow-growth mutants may have been diluted out because of their slower growth rate, artificially depressing their frequency in the cultures. However, we showed that plating at several doubling times beyond that required for phenotypic expression did not bias our results. Another possible explanation for the nonlinearity of the slow-growth phenotype may be the fact that the cultures treated at higher doses of insoluble compounds grew slower, and therefore, spent proportionately longer time exposed to nickel. This hypothesis is not likely since treatment with nickel sulfate also yielded a nonlinear response for the slow-growth phenotype, and we removed this soluble compound after treatment.

Differences in potency were observed in the toxic and mutagenic responses of TK6 cells to the nickel compounds. At high concentrations (50 mM) nickel oxide was not cytotoxic and did not induce the frequency of mutation. The other four nickel compounds (Ni_3S_2, $Ni(OH)_2$, $NiSO_4$, Ni metal) however, were cytotoxic at a much lower concentrations (95-1000 uM) and significantly induced slow-growing $tk^{-/-}$

mutants. This may be related to the bioavailability of the compound which affects the capacity of Ni(II) to reach a critical target site, such as nuclear proteins or DNA. Costa and Mollenhauer (1980) demonstrated that the carcinogenicity of particulate nickel compounds is directly proportional to the rate of cellular uptake. Uptake may vary significantly depending upon the chemical and physical properties of nickel compounds, including size, surface charge, and solubility (Kuehn and Sunderman, 1982). Increases in the concentrations of soluble nickel outside the cell have also been shown to inhibit phagocytosis and affect intracellular nickel levels (Costa and Heck, 1982, 1983).

5.2 Oxygen Radicals and Ni(II)-Induced Mutagenesis

The possible role of oxygen radicals in Ni(II)-induced mutagenesis was also investigated in the present study. Nickel has been shown to increase reactive oxygen species in mammalian cells (Huang et al., 1993) and to catalyze oxidation of DNA bases *in vitro* and *in vivo* (Nackerdien et al., 1991; Kasprzak and Hernandez, 1989; Kazpzak et al., 1992). Ni(II) is suspected to generate highly reactive oxygen radicals via the Fenton\Haber-Weiss reaction--including the superoxide radical (O_2^-), the hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) (for review, see Sunderman, 1989; Kawanishi et al., 1990). Oxygen radicals are known to cause several types of DNA damage including DNA strand breaks, DNA deletions, and chromosome aberrations (Hsie et al., 1986; Brawn and Fridovich, 1981).

The results of the present study, however, suggest that the induction of mutation by nickel compounds in human cells may not be mediated by oxygen radicals. The addition of H_2O_2 (20 uM) to nickel-treated cultures shifted the dose-response relationship for slow-growing $tk^{-/-}$ mutants to lower nickel concentrations, but the effect was less than a factor of two. We hypothesized that if Ni(II) were capable of catalyzing oxidative DNA damage in TK6 cells, a large increase in the mutant frequency would have been produced in the presence of Ni(II) and H_2O_2. It is possible that the concentration of H_2O_2 used in this study was not high enough to catalyze significant levels of Ni(II)-induced oxidative DNA damage. However, this concentration caused measurable toxicity (13%) and mutagenicity in TK6 cells, suggesting that the H_2O_2 exposures significantly perturbed the endogenous level of hydrogen peroxide.

Additional experiments in TK6 cells in the presence of the radical scavenger vitamin E (25 uM) were carried out to further examine the possible role of oxygen radicals in

Ni(II)-mediated mutation. Vitamin E has been demonstrated to act as a free-radical scavenger (Dean and Cheesman, 1987; Lieber et al., 1986; Summerfield and Tappel, 1984) and to inhibit the formation of chromosomal aberrations induced by crystalline nickel monosulfide particles (Lin et al., 1991). No significant decrease in mutation frequency was observed in our study by the addition of Vitamin E. The concentration of vitamin E used in these experiments was as high as could be used without reducing TK6 cell survival and significantly exacerbating the cytotoxicity caused by the nickel compounds.

However, evidence for vitamin E inhibition of Ni(II)-induced DNA damage and Ni(II)-catalysis of oxygen radicals is equivocal. Lin et al. (1991) did not evaluate nickel uptake in their study which may explain differences in carcinogenic potency observed in the absence and presence of vitamin E. Vitamin E is a lipophyllic membrane scavenger which may not be capable of reaching and associating with nuclear target proteins or DNA. Ambiguous results have also been reported for nickel and vitamin E-treated mammalian cells in gene locus studies (*gpt*) (Kargacin et al., 1993).

Also, in another recent study Kawanishi and associates observed that several nickel compounds readily induced DNA single and double strand breaks in human HeLa cells, but observed only a 1.5 fold increase over background of 8-OH-dG in nickel subsulfide and nickel sulfide treated cells (Kawanishi, unpublished). Strand breaks are likely to occur in the induction of large DNA alterations and slow-growing $tk^{-/-}$ mutants. Because the increase in strand breaks was proportionately much higher than the increase seen in oxidized DNA bases, these results suggest that the two endpoints may not related. Further research is needed to clarify the relationship between Ni(II)-induced oxidation of DNA bases *in vivo* and subsequent changes in mutation frequency.

The relatively small increase in mutation frequency observed by the addition of H_2O_2 to nickel-treated cells in our study may have been caused by Ni(II) inhibition of DNA excision repair. Ni(II) has been shown to inhibit repair of DNA damage caused by other mutagens (Swierenga et al., 1968; Hartwig and Beyersmann, 1988). Oxidative damage caused by added H_2O_2, therefore, may not have been efficiently repaired, resulting in the induction of more mutation in the presence of Ni(II) than in its absence.

6. Conclusions

Nickel exhibits both genotoxic and carcinogenic effects in humans, animals, and *in vitro* test systems, yet the mechanism by which nickel induces DNA damage and mutation remains unknown. Studies have shown that nickel binds weakly to DNA in solution, and is capable of causing single and double DNA strand breaks. Other evidence demonstrates that nickel binds readily to nuclear proteins, and may decrease the rate and fidelity of DNA replication and excision repair. Nickel has also been observed to catalyze oxidation of DNA bases *in vitro* and *in vivo*, suggesting that nickel may cause DNA damage by generating highly reactive oxygen radicals.

Evidence relating these genetic effects to the induction of mutation and cancer is equivocal. While nickel in the presence of H_2O_2 and DNA in solution clearly causes oxidative damage to DNA, these conditions may not be predictive of *in vivo* effects. In addition, the relative increase in nickel-induced oxidation *in vivo* (compared to background levels) is low compared to DNA damage at other endpoints, and to the level of genotoxicity and carcinogenicity observed in nickel-treated experimental animals.

Evidence of the relationship between nickel-induced mutagenesis and carcinogenesis via DNA replication and repair is also lacking. Nickel has been demonstrated to reduce the fidelity of DNA polymerase during replication, however, such a mechanism would most likely lead to point mutations and not the chromosome aberrations, sister chromatid exchanges, and deletions seen with nickel compounds. Excision repair studies have been contradictory, showing that nickel both inhibits and induces DNA repair.

While the mechanism of Ni(II)-induced genotoxicity remains unclear, our study clearly shows that the predominant mutagenic effect of nickel compounds is to induce large DNA alterations in human cells. These mutations may be large deletions, gene conversion, or reciprocal recombination. Nickel compounds also appear to be incapable of inducing point mutations, frameshifts, and small deletions. We conclude that the results of the present study suggest Ni(II)-induced mutagenesis may not be mediated by oxygen radicals.

7. Suggestions for Future Research

Future research on the mutagenicity of nickel compounds in TK6 cells should be directed toward understanding the mechanisms by which nickel-induces DNA damage and mutations. We suggest the following:

(I) Measure the rate of cellular uptake of particulate and soluble nickel compounds, as well as the affinity of nickel for chromatin and nuclear proteins in TK6 cells. This can be accomplished by using a radioactive isotope of nickel (^{63}Ni) to trace the intracellular distribution of nickel. Determine how the rate of uptake and binding vary as a function of dose, and see how these factors correlate to slow-growing $tk^{-/-}$ mutant fraction increases.

(II) Determine the type of DNA damage that is required to increase slow-growing $tk^{-/-}$ mutants in TK6 cells. This can be accomplished by quantifying the level of oxidized bases, and single and double DNA strand breaks, as a function of dose and see how this damage correlates to mutant fraction increases observed in slow-growing $tk^{-/-}$ mutants. TK6 cells should be synchronized to determine exactly when nickel-induced DNA damage occurs in the cell cycle.

(III) Investigate DNA replication and repair in TK6 cells. By knowing the rate of excision repair, as well as the enzymatic pathways of DNA replication in TK6 cells, we could have a better understanding of nickel-induced carcinogenesis.

8. References

Abbracchio, M.P., R.M. Evans, J.D. Heck, O. Cantoni, and M. Costa (1982a) The regulation of ionic nickel uptake and cytotoxicity by specific amino acids and serum components, Biol. Trace Element Res., 4, 219.

Abbracchio M.P., J.D. Heck, and M. Costa (1982b) The phagocytosis and transforming activity of crystalline metal sulfide particles is related to their negative surface charge, Carcinogenesis, 3,175-180.

Amacher, D.E. and S.C. Paillet (1980) Induction of trifluorothymidine-resistant mutants by metal ions in L5178Y/TK$^{+/-}$ cells, Mutat. Res., 78, 279.

Ames, B.N., M.C., Hollstein, and R. Cathcart (1982) In Lipid Peroxides in Biology and Medicine (K. Yagi, Ed.), Academic Press, New York, 339-351.

Ames, B.N. (1983) Dietary carcinogens and anticarcinogens; oxygen radicals and degenerative diseases, Science, 221, 1256-1264.

Asato, N., M. Van Soestbergen, and F.W. Sunderman, Jr. (1975) Binding of ^{63}Ni(II) to ultrafiltrable constituents of rabbit serum *in vivo* and *in vitro*, Clin. Chem., 21, 521.

Bhunya, S.P, and B.B. Pal (1986) Cytogenic effects of nickel fluride in mouse, The Nucleus, 29, 132-136.

Biggart, N.W. and M. Costa (1986) Assessment of the uptake and mutagenicity of nickel chloride in Salmonella tester strains, Mutat. Res., 175, 209.

Borek, C., A. Ong, H. Mason, L. Onahue and J.E. Biaglow (1986) Selenium and vitamin E inhibit radiogenic and chemically induced transformation *in vitro* via different mechanisms, Proc. Natl. Acad. Sci. (U.S.A.), 82, 6755-6759.

Brawn, K. and I. Fridovich (1981) DNA strand scission by enzymically generated oxygen radicals, Archives of Biochemistry and Biophysics, 206, 414-419.

Brommundt, G., and F. Kavaler (1987) La^{3+}, Mn^{2+}, and Ni^{2+} effects on Ca^{2+} pump and on Na^{+} - Ca^{2+} exchange in bullfrog ventricle, Am. J. Physiol., 253, C45-C51.

Clemente, G.F., L. Cigna Rossi, and G.P. Santaroni (1980) Nickel in foods and dietary intake of nickel. In: Nickel in the Environment (Nriagu, J.O., Ed.) New York, John Wiley and Sons, 493-498.

Cheng, K.C., D.S. Cahill, H. Kasai, S. Nishimura, and L.A. Loeb (1992) 8-Hydroxyguanosine, an abundant form of oxidative DNA damage, causes G to T and A to C substitutions, J.Biol. Chem., 267, 166-172.

Chovil, A., R.B. Sutherland, and M. Halliday (1981) Respiratory cancer in a cohort of nickel sinter plant workers, Br. J. Ind. Med., 38, 327-333.

Christie N.T., P. Sen, and M. Costa (1988) Chromosomal alterations in cell lines derived from mouse rhabdomyosarcomas induced by crystalline nickel sulfide, Biol. Metals, 1, 43-50.

Christie, N.T., D.M. Tummolo, C.B. Klein, and T.G. Rossman (1988) Role of Ni(II) in mutation. In: Nickel and Human Health, Current Perspectives: Advances in Environmental Sciences and Technology, Vol. 25 (E. Nieboer and J.O. Nriagu, Eds.), John Wiley and Sons, New York, 305-317.

Clary, J.J. (1975) Nickel choride-induced metabolic changes in the rat and guinea-pig, Toxicol. Appl. Pharmacol., 31, 55-65.

Ciccarelli, R.B., T.H. Hampton, and K.W. Jennette (1981) Nickel carbonate induces DNA-protein crosslinks and DNA strand breaks in rat kidney, Cancer Lett., 12, 349-354.

Ciccarelli, R.B, and K.E. Wetterhahn (1984) Nickel-bound chromatin, nucleic acids, and nuclear proteins from kidney and liver of rats treated with nickel carbonate *in vivo*, Cancer Res., 44, 3892-3897.

Conway, K., X.W., Wang, L.S. Xu, and M. Costa (1987) Effect of magnesium on nickel-induced genotoxicity and cell tranformation, Carcinogenesis, 8, 115-1121.

Coogan, T.P., D.M. Latta, E.T. Snow, and M. Costa (1989) Toxicity and carcinogenicity of nickel compounds, CRC Crit. Rev. Toxicol., 19(4), 341-84.

Costa M., H.H. Mollenhauer (1980a) Carcinogenic activity of particulate nickel compounds is proportional to their cellular uptake, Science, 209, 515-517.

Costa M., M.K. Jones, and O. Lindberg (1980b) Metal carcinogenesis in tissue culture systems. In: Inorganic Chemistry in Biology and Medicine, Vol. 140, Am. Chem. Soc. Smp., 45.

Costa, M., J. Simmons-Hansen, C.W.M. Bedrossian, J. Bonura, R.M. Caprioli, (1981) Phagocytosis, cellular distribution and carcinogenic activity of particulate nickel compounds in tissue culture, Cancer Res., 41, 2868-78.

Costa M., J.D., Heck, S.H. Robinson (1982) Selective phagocytosis of crystalline metal sulfide particles and DNA strandbreaks as a mechanism for the induction of cellular transformation, Cancer Res., 42, 2757-63.

Costa, M., J.D. Heck (1983) Influence of surface charge and dissolution on the selective phagocytosis of potentially carcinogenic particulate metal compounds, Cancer Res., 43, 5652-56.

Costa, M., (1989) Perspectives on the mechanism of nickel carcinogenesis gained from models of *in vitro* carcinogenesis, Environ. Health. Pers., 81, 73-76.

Costa, M., (1991) Molecular mechanisms of nickel carcinogenesis, Annu. Rev. Pharmacol. Toxicol., 31, 321-37.

Datta, A.K., M. Misra, S. L. North, and K.S. Kasprak (1992) Enhancement by nickel(II) and L-histidine of 2'-deoxyguanosine oxidation with hydrogen peroxide, Carcinogenesis,13, 283-287.

Dean, R.T., and K.H. Cheeseman (1987) Vitamin E protects proteins against free radical damage in lipid environment, Biochem. Biophys. Res. Commun., 148, 1277-1282.

DeLuca, J.G., L. Weinstein, and W.G. Thilly (1983) Ultraviolet light-induced mutation of diploid human lymphoblasts, Mutat. Res., 107, 347-370.

Deng, C., L.H. Horchang, X. Hanlong, Y. Mingchen, H. Jiachen, and O. Boaxiang (1988) Chromosomal aberrations and sister chromatid exchanges of periperal blood lymphocytes in Chinese electroplating workers: effect of nickel and chromium, The Journal of Trace Elements in Experimental Medicine, 1, 57-62.

DiPaolo, J.A. and B.C., Castro (1979) Ouantitative studies of *in vitro* morphological transformation of Syrian hamster fetal cells by inorganic metal salts, Cancer Res., 39, 1008-1013.

Doll, R., J.D. Mathews, and L.G. Morgan (1977) Cancers of the lung and nasal sinuses in nickel workers: A reassessment of the period of risk, Br. J. Ind. Med., 34, 102-105.

Doll, R., (1983) Nickel exposure: a human health hazard, IARC document, 3-21.

Donskoy, E., M. Donskoy, F. Faripour, C.G. Gillies, A. Marzouk, M.C. Reid, O. Zaharia, and F.W. Sunderman, Jr. (1986) Hepatic toxicity of nickel chloride in rats, Ann. Clin. Lab. Sci., 16, 108-117.

Eichhorn, G.L. (1962) Metal ions as stabalizers or destabilizers of deoxyribonucleic acid structure, Nature (London), 194, 474-475.

Eichhorn, G. L., and Y.A. Shin (1968) Interaction of metal ions with polynucleotides and related compounds. XII. The relative effect of various metal ions on DNA helicity, J. Am. Chem. Soc., 90, 7323-7328.

Evans, R.M., P.J.A. Davies, and M. Costa (1982) Video time-lapse microscopy study of phagocytosis and interacellular fate of crystalline nickel sulfide particles in cultured mammalian cells, Cancer Cells, 42, 2729-35.

Gebhart, E., H. Wagner, K. Griziwok and H. Behnsen (1985) The action of anticlastogens in human lymphocyte cultures and their modicfication by rat-liver S9 mix, Studies with vitamins C and E, Mutat. Res., 149, 83-94.

Grandjean, P. (1984) Human exposure to nickel. In: Nickel in the Human Environment, IARC Monogr. Ser. No. 53 (Sunderman, F.W., Jr., Ed.) IARC Scientific Publications, Lyon, France, 460.

Green, M.H.L., W.J. Muriel and B.A. Bridges (1976) Use of a simplified fluctuation test to detect low levels of mutagens, Mutat. Res., 38, 33-42.

Hamilton-Koch W., R.D. Snyder, J.M. LaVelle (1985) Characterization of DNA damage and repair induced by metal compounds in cultured human fibroblasts, Toxicologist, 5, 23.

Hansen, K. and R.M. Stern (1984) Toxicity and tranformation potency of nickel compounds in BHK cells *in vitro*. In Sunderman, F.W., Jr. (ed.in-chief), Nickel in the Human Environment. Oxford University Press, Oxford, 193-199.

Harnet, P.B., S.H. Robison, D.E. Swartzendruber, and M. Costa (1982) Comparison of Protein, RNA, and DNA Binding and cell-cycle specific growth inhibitory effects of nickel compounds in cultured cells, Toxicol. Appl. Pharmacol., 64, 20-30.

Hartwig, A., and D. Beyersmann (1985) Chromium and nickel compounds: mutagenicity and inhibition of DNA repair. In: Abstracts of the first international meeting on molecular mechanisms of metal toxicity and carcinogenesis (Costa M., Ed.) Urbino (Italy): Collegio Universitario, 116.

Heck, J.D., and M. Costa (1982) Extracellular requirments for the endocytosis of carcinogenic crystalline nickel sulfide particles by facultative phagocytes, Toxicol. Letter, 12, 243-250.

Hsie, A.W., L. Recio, D.S. Katz, C.Q. Lee, M. Wagner, and R.L. Schenley (1986) Evidence for reactive oxygen species inducing mutations in mammalian cells, Proc. Natl. Acad. Sci., 83, 9616-9620.

Huang, Xi, K. Frenkel, C.B. Klein, and M. Costa (1993) Nickel induces increase in oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence, Toxicol. Appl. Pharmacol., 120, 29-36.

Hui, G., and F.W. Sunderman, Jr. (1980) Effects of nickel compounds on incorporation of (^{3}H) thymidine into DNA in rat liver and kidney, Carcinogenesis,1, 297-304.

IPCS, International Programme on Chemical Safety (1991), Enivronmental Health Criteria 108, Nickel (1991), World Health Organization, Geneva.

Jasmin, G. and J.L. Riopelle (1976) Renal carcinomas and erythocytosis in rats following intrarenal injection of nickel subsulfide, Lab. Invest., 35, 71-78.

Jenkins, D.W. (1980) Nickel Accumulation in Aquatic Biota. In: Nickel in the Environment (Niragu, J.O., Ed.) John Wiley and Sons, New York, 283.

Kalinina, L.M., G.M. Sardarly and U.K. Alekperov (1979) Antimutagenic effect of alpha-tocopherol on the frequency of gene mutations in Salmonella, Benetika, 15, 1880-1888.

Kargacin, B., C.B. Klein, and M. Costa (1993) Mutagenic responses of nickel oxides and nickel sulfides in Chinese hamster V79 cell lines at the xanthine-guanine phosphoribosyl transferase locus, Mutat. Res., 300, 63-72.

Kasprzak, K.S. and L.A. Poirier (1985) Effects of calcium (II) and magnesium (II) on nickel (II) uptake and stimulation of thymidine incorporation into DNA in the lungs of strain A mice, Carcinogenesis, 6, 12, 1819-1821.

Kasprzak, K.S., M.P. Walkes, and L.A. Poirier (1986) Antagonism by essential divalent metals and amino acids of nickel (II)--DNA binding *in vitro*, Toxicol. Appl. Pharmacol., 82, 336-342.

Kazprzak, K.S. and L. Hernandez (1989) Enhancement of hycroxylation and deglycosylation of 2'-deoxyguanosine by carcinogenic nickel compounds, Cancer Res., 49, 5964-5968.

Kasprzak, K.S., B.A. Diwan, J.M. Rice, M. Misra, C.W. Riggs, R. Olinski, and M. Dizdaroglu (1992) Nickel (II)-mediated oxidative DNA base damage in renal and hepatic chromatin of pregnant rats and their fetuses. Possible relevance to carcinogenesis, Chem. Res. Toxicol., 5, 809-815.

Kavaler, F., and G. Brommundt (1987) Potentiation of contraction in bullfrog ventricle strips by manganese and nickel, Am. J. Physiol., 253, C52-C59.

Kawanishi, S., S. Inoue, K. Yamamoto (1989) Site-specific DNA damage induced by nickel (II) ion in the presence of hydrogen peroxide, Carcinogenesis, 10, 12, 2231-2235.

Kawanishi, S. (unpublished) Oxygen radical formation and DNA damage induced by nickel compounds In relation to carcinogenicity.

Kodama, Y., C.J. Boreiko, T. R. Skopek, and L. Recio (1989) Cytogenic analysis of spontaneous and 2-cyanoethylene oxide-induced tk-/- mutants in TK6 human lymphoblastoid cultures, Environmental and Molecular Mutagenesis, 14, 149-154.

Kuehn, K., C.B. Fraser, F.W. Sunderman, Jr. (1982) Phagocytosis of particulate nickel compounds by rat peritoneal macrophages *in vitro*, Carcinognesis, 3, 321-326.

Kuehn, K., and F.W. Sunderman Jr., (1982) Dissolution half-times of nickel compounds in water, rat serum, and renal cytosol, Journal of Inorganic Biochemistry, 17, 29-39.

Larramendy, M.L., N.C. Popescu, and J.A. DiPaolo (1981) Induction by inorganic metal salts of sister chromatid exchanges and chromosome aberrations in human and Syrian hamster cell strains, Environ. Mut., 3, 597-606.

Liber, H.L., and W.G. Thilly (1982) Mutation assay at the thymidine kinase locus in diploid human lymphoblasts, Mutat. Res., 94, 467-485.

Liber H.L., D.W. Yandell, and J.B. Little (1989) A comparison of mutation induction at the tk and hprt loci in human lymphoblastoid cells; quantitative differences are due to an additional class of mutations at the autosomal *tk* locus, Mutat. Res., 216, 9-17.

Lieber, D.C., D.S. Klings and D.J. Read (1986) Antioxidant protection of phospholipid bilayers by alpha-tocopherol: control of alpha-tocopherol status and lipid perosication of ascorbic acid and glutathione, J. Biol. Chem., 261, 12114-12119.

Lin, X., M. Sugiyama, and M. Costa (1991) Differences in the effect of vitamin E on nickel sulfide or nickel chloride-induced chromosomal aberrations in mammalian cells, Mutation Res., 260, 159-164.

Lucassen, M. and B. Sarkar (1979) Nickel (II) binding constituents of human blood serum, J. Toxicol. Environ. Health, 5, 897.

Magnus, K., A. Andersen, and A.C. Hogetveit (1982) Cancer of respiratory organs among workers at a nickel refinery in Norway, Int. J. Cancer, 30, 681-85.

Marzilli, L.G. (1981) Metal complexes of nucleic acid derivatives and nucleotides: Binding sites and structures. In: Metals Ions in Genetic Information Transfer (G.L. Eichhorn and L.G. Marzilli, Eds.) Elsevier/North Holland, New York, 48-81.

Mastromatteo E (1986) Nickel, Am. Ind. Hyg. Assoc. J., 47, 589-601.

Mastromatteo E. (1988) Nickel and its compounds. In: Occupational medicine: Principles and practical applications, second edition (Zenz, C., Ed) Year Book Medical Publishers, Chicago, 597-608.

Medinsky, M.A., J.M. Benson, and C.H. Hobbs (1987) Lung clearance and disposition of the ^{63}Ni in F344/N rats after intratracheal instillation of nickel sulfate solutions, Environ. Res., 43, 168178.

Miyaki, M., I. Murata, M. Osabe, and T. Ono (1977) Effect of metal cations on misincorporation by E. Coli DNA polymerases, Biochem. Biophys. Res. Commun., 77, 854-860.

Miyaki, M., N. Akamatsu, T. Ono, and H. Koyama (1979) Mutagenicity of metal cations in cultured cells from Chinese hamster, Mutat. Res., 68, 259.

Montaldi, A., L. Zentilin, M. Zordan, V. Bianchi, and A.G. Levis (1987) Chromosomal effects of heavy metals (Cd, Cr, Hg, Ni and Pb) on cultured mammalian cells in the presence of nitrilotriacetic acid (NTA), Toxicological and Environmental Chemistry, 14, 183-200.

Morgan, J.G. (1958) Some observations on the incidence of respiratory cancer in nickel workers, Br. J. Ind. Med., 15, 224-234.

Nackerdien, Z., K.S. Kasprzak, G. Rao, B. Halliwell, and M. Dizdaroglu (1991) Nickel (II) and cobalt (II)-dependent damage by hydrogen peroxide to the DNA bases in isolated human chromatin, Cancer Res., 51, 5837-5842.

Nishimura, M., and M. Umeda (1978) Mutagenic effect of some metal compounds on cultured mammalian cells, Mutat. Res., 54, 246-247.

Nishimura, M., and M. Umeda (1979) Induction of chromosomal aberrations in cultured mammalian cells by nickel compounds, Mutat. Res., 68, 337-349.

Nomoto, S., M.D. McNeely, and F.W. Sunderman, Jr. (1971) Isolation of a nickel alpha$_2$-macroglobulin from rabbit serum, Biochemistry, 10, 1647.

Newman, S.M., R.L. Summit, and L.J. Nunez (1982) Incidence of nickel-induced sister-chromatid exchange, Mutat. Res., 101, 67-75.

Nieboer, E., R.I. Maxwell, and A.R. Stafford (1984) Chemical and biological reactivity of insoluble nickel compounds and the bioinorganic chemistry of nickel, in Nickel in the Human Environment, IARC Monogr. Ser. No. 53, Sunderman, F.W., Jr., Ed., IARC Scientific Publications, Lyon, France, 439.

Nieboer, E., R.I. Maxwell, F.E. Rossetto, A.R. Stafford, P.I Stetsko (1986) Concepts in nickel carcinogenesis. In: Frontiers in Bioinorganic Chemistry (Xavier, A.V., Ed.) VCH Verlagsgesellschaft mbH, Weinheim, Federal Republic of Germany, 142-151.

Nieboer, E., R.T. Tom, W.E. Sanford (1988) Nickel metabolism in man and animals. In: Nickel and its Role in Biology, Vol. 23, Metal Ions in Biological Systems (Sigel, H., Ed.) Marcel Dekker, New York 91-121.

Nriagu, J. O. (1980) Global Cycles and Properties of Nickel. In: Nickel in the Environment, John Wiley and Sons, New York, 1--26.

Okamoto, M. (1987) Induction of ocular tumor by nickel subsulfide in the Japanese common newt, *Cynops pyrrhogaster*, Cancer Res., 47, 5213.

Ottolenghi, A.D., J.K. Haseman, W.W. Payne, H.L. Falk, and H.N. MacFarland (1974) Inhalation studies of nickel sulfide in pulmonary carcinogenesis of rats, J. Natl. Cancer Inst., 54, 1165.

Patierno, S.R., and M. Costa (1985) DNA-protein cross-links induced by nickel compounds in intact cultured mammalian cells, Chem. Biol. Interactions, 55, 75-91.

Pedersen, E., A.C. Hogetveit, and A. Andersen (1973) Cancer of respiratory organs among workers at a nickel refinery in Norway, Int. J. Cancer, 12, 32-41.

Pikalek, P., and J. Necasek (1983) The mutagenic activity of nickel in *Corynebacterium* sp., Folia Microbiol., 28, 17.

Radner, B.S., and A.R. Kennedy (1986) Suppression of X-ray induced transformation by vitamin E in mouse C3H/10T1/2 cells, Cancer Lett., 32, 25-32.

Roberts, R.S., J.A. Julian (1982) Mortality study of Canadian nickel miners. In: Wagner, W.L., W.N. Rom, J.A. Merchant, eds. Proceedings of the conference on health issues related to metal and non-metallic mining, Park City, UT, 241-260.

Robison, S.H., O. Cantoni, M. Costa, (1982) Strand breakage and decreased molecular weight of DNA induced by specific metal compounds, Carcinogenesis, 3, 657-662.

Robison S.H., O. Cantoni, J.D. Heck, M. Costa (1983) Soluble and insoluble nickel compounds induce DNA repair synthesis in cultured mammalian cells, Cancer Lett., 17, 273-279.

Robison S.H., O. Cantoni, M. Costa (1984) Analysis of metal induced DNA lesions and DNA-repair replication in mammalian cells, Mutat. Res., 131, 173-183.

Schmidt, J.A. and A. W. Andren (1980) The atmospheric chemistry of nickel. In: Nickel in the Environment (Niragu, J.O., Ed.) John Wiley and Sons, New York, 93-136.

Sen, P., and M. Costa (1986) Incidence and localization of sister chromatid exchanges induced by nickel and chromium compounds, Carcinogenesis,7, 1527-1533.

Senft, V., L. Frantisek, and M. Tucek (1992) Cytogenic analysis of chromosomal aberrations of peripheral lymphocytes in workers occupationally exposed to nickel, Mutat. Res., 279, 171-179.

Shibutani, S., M. Takeshita, and A.P. Grollman (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-OH-dG, Nature, 349, 431-434.

Sigel, Helmut, and Astrid Sigel (1988) Metal Ions in Biological Systems. In: Nickel and its Role in Biology, Vol. 23, Marcel Dekker, Inc., New York and Basel,

Singh, I. (1983) Induction of reverse mutation and mitotic gene conversion by some metal compounds in *Saccharomyces cerevisiae*, Mutat., Res., 117, 149-152.

Sirover, M.A., and L.A. Loeb (1976) On the fidelity of DNA replication, Journal of Biological Chem., 252, 3605-3610.

Skopek, T.R., H.L. Liber, D.A. Kaden, and W.G. Thilly (1978) Relative sensitivites of forward and reverse mutation assays in *Salmonella typhimurium*, Proc. Natil. Acad. Sci. (USA), 75, 4465-4469.

Sobti, R.C. and R.K. Gill (1989) Incidence of micronuclei and abnormalities in the head of spermatozoa caused by the salts of a heavy metal, nickel, Cytologia, 54, 249-253.

Stinson, T.J., S. Jaw, E.H. Jeffery, and M.J. Plewa (1992) Toxicol. Appl. Pharmacol., 117, 98-103.

Sunderman, Jr., F.W. and R.M. Maenza (1976) Comparisons of carcinogeniciities of nickel compounds in rats. Res. Commun. Chem. Pathol. Pharmacol., 14, 319-330.

Sunderman, R.W., Jr. 91981) Recent research on nickel carcinogenesis, EHP Environ. Health Perspect., 40, 131-141.

Sunderman, F.W., Jr. (1988) Pulmonary toxicity induced in rats by parenteral administration of nickel chloride, paper presented at workshop on Toxic Metal Compounds. International Association of Environmental and Analytical Chemistry, Collonica, Italy, April 10-14.

Sunderman, F.W. (1984) Nickel in the Human Environment. International Agency for Research on Cancer, Lyon, France, Preceedings of a joint symposium, March 8-11, 1983.

Sunderman, F.W., Jr., A. Marzouk, S.M. Hopfer, O. Saharia, and M.C. Reid (1985) Increased lipid peroxidation in tissues of nickel chloride-treated rats, Ann., Clin. Lab. Sci, 15, 229-236.

Sunderman, F.W., Jr. (1987) Lipid peroxidation as a mechanism of acute nickel toxicity, Toxicol. Environ. Chem., 15, 59-69.

Sunderman, F.W., Jr., S.M. Hopfer, J.A, Knight, K.S. McCully, A.G. Cecutti, P.G. Thornhill, K. Conway, C. Miller, S. R. Patierno, and M. Costa (1987) Physicochemical characteristics and biological effects of nickel oxides, Carcinogenesis, 8, 305-313.

Sunderman, F.W., Jr. (1989) Mechanisms of Nickel Carcinogenesis, Scand. J. Work. Environ. Health, 15, 1-12.

Sunderman, W.F. Jr., S.M. Hopfer, W.W. Nichols, J.R. Selden, H. L. Allen, C.A. Anderson, R. Hill, C. Bradt, and C.J. Williams (1990) Chromosomal abnormalities and gene amplification in renal cancers induced in rats by nickel subsulfide, Annals of Clinical and Laboratory Science, 20, 60-72.

Swierenga, S.H.H., P.K. Basrur (1968) Effect of nickel on cultured rat embryo muscle cells, Lab. Invest., 19, 663-674.

Swierenga, S.H.H., and J.R. McLean (1985) Further insights into mechanisms of nickel-induced DNA damage: studies with cultured rat liver cells. In: Progress in Nickel Toxicology (Brown, S.S. and Sunderman, F.W., Jr., Eds.) Blackwell Scientific, Oxford, 101.

US EPA (1986) Health assessment document for nickel and nickel compounds, Office of Health and Environmental Assessment, Washington, DC, 20460, EPA/600/8-83/012FF.

US NIOSH (National Institute of Occupational Safety and Health) (1977) Criteria for a recommended standard. Exposure to Inorganic Nickel. Washington, DC: DHEW (NIOSH). Publication 77-164.

Van Soestbergen, M., and F.W. Sunderman, Jr. (1972) $^{63}Ni(II)$ complexes in rabbit serum and urine after injection of $^{63}NiCl_2$, Clin. Chem., 1478.

Warner, J.S. (1984) Occupational exposure to airborne nickel in producing and using primary nickel products. In: Nickel in the Human Environment, Proceedings of a Joint Symposium, Lyon, 8-11 March, 1983, Lyon, International Agency for Research on Cancer, 419-437 (IARC Scientific Publications No. 53).

Wehner, A.P., and D.K. Craig (1972) Toxicology of inhaled NiO and CoO in Syrian golden hamsters, Am. Ind. Hyg. Assoc. J., 33, 146-155.

Yandell, D.W., T.P. Dryja, and J.B. Little (1986) Somatic mutations at a heterozygous autosomal locus in human cells occur more frequently by allele loss than by intragenic structural alterations, Somatic Cell and Molecular Genetics, 12, 255-263.

Yandell, D.W., T.P. Dryja, and J.B. Little (1990) Molecular genetic analysis of recessive mutations at a heterzygous autosomal locus in human cells, Mutat. Res., 89-102.

9. Appendix of Tables and Figures

Table 1: Chemical and Physical Properties of Nickel Compounds

Compound	Chemical Formula	Relative Molecular Mass	Density	Melting Point (C)	Boiling Point (C)	Solubility (water; other solvent)
Nickel	**Ni**	**58.7**	**8.9**	**1555/1455**	**2837**	***insoluble***
Nickel acetate	Ni(CH3CO2)2	176.8	1.744	—	—	*soluble; soluble in alcohol*
Nickel arsenate	Ni3(AsO4)2	453.97	4.982	—	—	*insoluble; soluble in acids*
Nickel bromide	NiBr2	218.53	—	—	—	*soluble; soluble in alcohol*
Nickel carbonate	2NiCO3	118.7	—	decomposes	—	*insoluble; soluble in acids*
Nickel carbonyl	Ni(CO)4	170.73	1.318 (17 C)	-19.3	43	*insoluble; soluble in organic solvents*
Nickel chloride	NiCl2	129.61	3.55	—	987	*soluble*
Nickel fluoride	NiF2	96.69	4.72	—	—	*slightly soluble*
Nickel hydroxide	**Ni(OH)2**	**92.72**	**---**	**decomposes <200**	**---**	***insoluble; soluble in acids/ammonia***
Nickel nitrate	Ni(NO3)2	182.72	2.05	56.7	137	*soluble; soluble in alcohol*
Nickel oxide	**NiO**	**74.69**	**6.67**	**1990**	**---**	***insoluble; soluble in acid***
Nickel phosphate	Ni3(PO4)3	366.07	—	—	—	*insoluble; soluble in acid*
Nickel sulfate	**NiSO4**	**154.77**	**---**	**53.3**	**---**	***soluble***
Nickel sulfide	NiS	90.77	5.3	797	—	*insoluble*
Nickel subsulfide	**Ni3S2**	**240.26**	**5.82**	**790**	**---**	***insoluble; soluble in nitric acid***

Table 2: World Nickel Production (1991)

▪ Ex-USSR	275,000
▪ Canada	120,300
▪ Japan	114,200
▪ Norway	58,700
▪ Australia	49,400
▪ New Caledonia	34,400
▪ China	30,000
▪ Dominican Republic	29,100
▪ United Kingdom	28,600
▪ South Africa	26,900
▪ Colombia	20,200
▪ Cuba	18,800
▪ Zimbabwe	18,200
▪ Greece	16,000
▪ Finland	13,800
▪ Brazil	13,400
▪ South Korea	11,400
▪ Taiwan	11,200
▪ France	8,700
▪ USA	7,100
▪ Indonesia	5,300
▪ ex-Yugoslavia	3,500
▪ Austria	3,500
▪ Czechoslovakia	3,500
▪ Albania	800
▪ **Total**	**920,900**

Source: UNEP IE/PAC, Tech. Rept. 15, 1993

Table 3: Occupations with Potential Exposure to Nickel Compounds

- Battery makers, storage
- Cashiers
- Catalyst workers
- Cement-carbide workers
- Ceramic makers
- Disinfectant makers
- Dyers
- Electroplaters
- Enamellers
- Gas-mask makers
- Glass makers
- Ink makers
- Jewellers
- Magnet makers
- Metalizers
- Mond-process workers
- Nickel-alloy makers
- Nickel miners
- Nickel refiners
- Nickel smelters
- Oil dehydrogenators
- Organic chemical synthesizers
- Paint makers
- Penpoint makers
- Petroleum refinery workers
- Spark plug makers
- Stainless steel makers
- Textile dyers
- Vacuum tube makers
- Varnish makers
- Welders

Source: Adapted from US NIOSH (1977)

Table 4: Organ Distribution of Nickel

Investigator	Species	Compound	Dose	Route	Time	Distribution
Jasim and Tjalve, 1984, 1986	Mouse	Nickel Chloride	10 umol Ni/kg BW	p.o.	5h	Kidney, lung, liver, heart, eyes, pancreas = fat = spinal cord, brain
					24h	Kidney, lung, spinal cord = heart = eyes = fat, liver, pancreas, brain
					72h	Eyes, kidney = spinal cord, liver = lung, heart, pancreas = fat, brain
	Fetus (18 -d)				24h	Placenta, Kidney, heart, lung, liver, brain
Sunderman et al. 1978	Rat	Nickel Chloride	12 mg Ni/kg BW	i.m.	24h	Kidney, adrenal = lung = ovary = uterus, spleen = heart = liver, muscle, pituitary
	Pregnant day 9					Kidney, fetus = adrenal glands = lung= ovary, pituitary, spleen = heart = liver, muscle
	day 19					Kidney, placenta = lung, adrenal, fetus, ovary = pituitary, sleen = heart, liver, muscle
Sarkar, 1981	Rat	Nickel Chloride	82 ug/kg BW	i.p.	6h	Kidney, spleen, lung, heart, liver, muscle
					18h	Kidney, lung, spleen = heart, muscle
					24 h	Kidney, lung, spleen = heart, liver, muscle
Baselt and Hanson, 1982	Rat	Nickel Carbonyl	1.1 mg/l, 210, 15 min	inhal.	24	Lung, heart, kidney = brain, liver
Carvalho and Ziemer, 1982	Rat	Nickel Chloride	1.27 ug Ni	i.t.	35 min	Lung,kidney, heart, skin, bone = spleen, liver = testis
					1 d	Lung, kidney, bone, spleen, heart, liver = testis, skin
					3 d	Lung, kidney, bone = spleen = skin, heart = liver = testis
					7 d	Lung, kidney = spleen, bone
					21 d	Lung, kidney
	Rat pregnant 12 and 19 d	Nickel Chloride	4 mg/kg BW	i.p.	15 min – 24	Kidney, pancreas, hypothsis, lung = placenta = liver, overy, fetus
Mas et al., 1985, 1986	Rat	Nickel Acetate	95 umol Ni/kg BW	i.p.	24 h	Kidney, lung = pancreas, pituitary, spleen, liver, heart, brain
Waalkes et al., 1985; Kasprzak et al., 1986	Rat	Nickel Sulfate	1.8 umol Ni	i.t.	4 h	Lung, kidney, trachea, adrenal glands, larynx = large intestine = thyroids = urinary bladder
Medinsky et al., 1987					24 h	Lung, kidney = trachea, larynx = large intestine = thyroids = urinary bladder, adrenal glands
					96 h	Lung, kidney, trachea = urinary bladder larynx = large intestine = thyroids, adrenal glands

Source: Coogan et al., 1989

Table 5: Dissolution Half Times (T50) for Nickel Compounds in Water, Rat Serum, and Renal Cytosol

Compound	Water (T50)	Rat Serum (T50)	Renal Cytosol (T50)
Nickel dust	> 11 yr	> 11 yr	8.4 yr
Nickel oxide	> 11 yr	> 11 yr	> 11 yr
Nickel disulfide	96 days	a	a
Nickel monosulfide	3.3 yr	2.6 yr	1.4 yr
Nickel monosulfide (amorphous)	34 days	24 days	19 days
Nickel subsulfide	10.4 yr	34 days	21 days
Nickel monoselenide	7.3 yr	1.1 yr	161 days
Nickel subselenide	> 11 yr	50 days	88 days
Nickel telluride	> 11 yr	7.9 yr	171 days
Nickel sulfarsenide	1.0 yr	1.0 yr	1.1 yr
Nickel monoarsenide	> 11 yr	46 days	14 days
Nickel subarsenide	> 11 yr	246 days	20 days
Nickel subarsenide	> 11 yr	73 days	110 days
Nickel antimonide	> 11 yr	> 11 yr	> 11 yr
Nickel ferrosulfide	10.8 yr	4.5 yr	329 days
Ferronickel alloy	> 11 yr	> 11 yr	> 11 yr
Nickel titanate	> 11 yr	> 11 yr	> 11 yr

[a] Flocculent precipitates formed during incubation of NiS_2 in rat serum and renal cytosol.

Source: Kuehn and Sunderman, 1982.

Table 6: Genotoxicity of Nickel Compounds

Endpoint	Species/Test System	Marker	Compound	Result	Reference
Gene Mutation					
Prokaryote	S. typhimurium	his	NiCl2, NiSO4	+/-	Arlauskas et al. (1985); Lavelle and Wilmer (1981)
	S. typhimurium	his	NiCl2	-	Biggart and Costa (1986)
	E. coli	trp	NiCl2	-	Green et al. (1986)
	Corynebacterium sp.		NiCl2	+	Pikalek and Necasek (1983)
Eukaryote	S. cerevisiae	trp, ilv	NiSO4	-	Singh (1983)
Mammalian Cells	CHO cells	hprt	NiCl2	?	Hsie et al. (1979)
	CHO cells		Ni3S2	+	Costa et al. (1980)
	Mouse lymphoma	tk	NiCl2	+	Amacher and Paillet (1980)
	V79 hamster cells	hprt	NiCl2	?	Miyaki et al. (1980)
	Rat kidney cells	ts-sarc	NiCl2	+	Biggart et al (1987); Biggart and Murphy (1988)
	V-79	gpt	NiSO4, NiS	+	Christie and Tummulo (1988)
Human Cells	Human foreskin firbroblasts		Ni3S2	-	Biedermann and Landolph (1987)
DNA damage	B. subtilis		NiO	-	Kanematsu et al. (1980)
	CHO cells		NiCl2, NiS	+	Ohno et al. (1982)
SCE	CHO cells		NiCl2, NiS	+	Sen and Costa (1986)
	Human lymphocytes		NiCl2	+	Newman et al. (1982)
Chromosome Aberrations	Hamster cells		NiCl2, NiSO4	+	Sen and Costa (1986); Larramendy et al. (1981)
	Human lymphocytes		NiSO4	+	Larramendy et al. (1981)
	Human bronchial epithelial cells		NiSO4	+	Lechner et al. (1984)
	FM3A mammary carcinoma cells		NiCl2, Ni3S2	+/-	Nishimura and Umeda (1979)
Cell Transformation	Hamster cells and C3H/10T/2 cells		NiCl2, Ni3S2, NiO	+	Dipaolo and Casto (1979); Hansen and Stern (1982, 1984)
	BHK-21 cells		NiO, Ni2O3, Ni3S2	+	Costa and Heck (1982); Saxholm et al. (1981)

Table 7: Nickel Sulfate/ Experiment 1

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	146	384	100.00	96	276	110
20	138	384	105.83	74	231	44
40	130	384	112.00	95	228	100
60	170	382	83.72	42	195	26
0 + H2O2	216	384	59.50	56	201	129
20 + H2O2	197	382	68.50	77	209	89
40 + H2O2	198	384	68.50	39	247	102
60 + H2O2	230	384	53.00	131	182	134

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	14	384	60	192	58.16	1.60	39	384	4.60	15	384	65	192	54.16	1.84
20	14	384	39	192	79.70	1.17	42	384	3.63	5	384	73	190	47.83	0.69
40	15	383	47	192	70.37	1.42	35	384	3.40	14	384	55	192	62.51	1.49
60	10	384	40	192	78.43	0.84	44	384	3.88	8	384	25	192	101.93	0.52
0 + H2O2	16	384	50	192	67.27	1.58	54	384	5.63	27	384	70	192	50.45	3.61
20 + H2O2	20	384	46	192	71.44	1.87	52	384	5.09	28	384	33	190	87.53	2.16
40 + H2O2	11	384	41	192	77.20	0.94	65	384	6.01	24	384	52	192	65.31	2.47
60 + H2O2	38	384	54	192	63.43	4.11	52	384	5.74	38	384	56	192	61.61	4.23

Table 8: Nickel Sulfate/ Experiment 2

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	99	384	100.00	338	320	295
40	102	384	97.80	326	234	358
60	121	384	85.20	240	200	371
95	151	384	68.86	213	238	311
0 + H2O2	161	384	64.13	312	201	183
40 + H2O2	151	384	68.86	336	273	324
60 + H2O2	150	384	69.35	374	275	302
95 + H2O2	237	384	35.60	158	328	226

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	64	384	38	192	81.00	5.63	61	384	5.34	63	384	31	192	91.18	4.91
40	65	383	36	192	83.70	5.56	48	384	3.99	71	384	36	192	83.70	6.11
60	60	381	31	192	91.18	4.70	51	384	3.91	84	384	35	192	85.11	7.25
95	53	376	44	192	73.67	5.16	60	384	5.77	82	384	39	192	79.70	7.54
0 + H2O2	103	384	28	192	96.26	8.11	70	384	5.23	73	384	21	192	110.65	4.76
40 + H2O2	90	383	37	192	82.33	8.13	75	383	6.62	85	384	39	192	79.70	7.85
60 + H2O2	97	384	38	192	81.00	8.99	74	384	6.61	77	384	41	192	77.20	7.25
95 + H2O2	71	383	48	192	69.31	7.39	133	384	15.34	110	384	39	192	79.70	10.59

Table 9: Nickel Sulfate/ Experiment 3

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	73	382	100.00	49	224	377
95	160	384	52.90	69	182	147
145	263	384	22.87	31	165	63
175	309	384	13.13	16	91	29
0 + H2O2	124	384	68.30	79	274	197
75 + H2O2	181	384	45.45	49	168	213
95 + H2O2	259	384	23.80	38	147	81
110 + H2O2	280	384	19.09	37	190	62

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	11	384	32	192	89.59	0.81	48	384	3.73	64	384	45	192	72.54	6.28
95	27	384	36	192	83.70	2.18	67	384	5.73	55	384	36	192	83.70	4.62
145	25	384	42	190	75.47	2.23	117	384	12.04	54	384	37	192	82.33	4.60
175	24	384	40	192	78.43	2.06	117	384	11.58	46	384	33	192	88.05	3.62
0 + H2O2	24	384	36	192	83.70	1.93	77	384	6.68	50	384	45	192	72.54	4.81
75 + H2O2	19	384	47	192	70.37	1.80	61	384	6.15	87	384	37	192	82.33	7.80
95 + H2O2	33	384	35	190	84.58	2.66	113	384	10.30	73	384	30	192	92.81	5.68
110 + H2O2	37	384	40	192	78.43	3.23	156	384	16.62	64	384	36	192	83.70	5.45

Table 10: Nickel Sulfate/Experiment 4

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	90	384	100.00	61	165	248
95	252	384	29.03	16	101	48
145	352	384	6.00	3	27	8
175	372	383	2.01	2	14	3
0 + H2O2	119	384	80.75	90	208	182
75 + H2O2	219	384	38.71	25	208	45
95 + H2O2	278	384	22.27	29	110	32
110 + H2O2	287	384	20.07	21	167	25

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	11	384	46	192	71.44	1.02	29	384	2.75	43	366	42	191	75.73	4.13
95	11	384	38	192	81.00	0.90	66	384	5.82	31	381	41	192	77.20	2.75
145	11	384	31	192	91.18	0.80	93	384	7.60	22	383	47	192	70.37	2.10
175	18	384	36	192	83.70	1.43	119	379	11.26	30	384	44	192	73.67	2.76
0 + H2O2	17	384	57	192	60.72	1.86	38	384	4.29	36	382	51	190	65.76	3.76
75 + H2O2	11	384	51	192	66.28	1.10	81	384	8.94	21	381	44	192	73.67	1.92
95 + H2O2	22	384	50	191	67.01	2.20	76	384	8.23	27	368	40	192	78.43	2.43
110 + H2O2	17	384	54	192	63.43	1.78	114	384	13.88	22	373	44	192	73.67	2.06

Table 11: Nickel Subsulfide/Experiment 1

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	203	384	100.00	78	276	197
70	226	384	83.16	79	251	123
140	324	384	26.65	33	151	46
210	375	384	3.72			
0 + H2O2	280	384	49.55	143	315	316
70+ H2O2	330	384	23.78	70	226	140
140+ H2O2	380	384	1.64			

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	10	384	70	192	50.45	1.31	34	384	4.59	40	384	36	192	83.70	3.29
70	12	384	70	192	50.45	1.57	37	384	5.02	29	384	39	192	79.70	2.46
140	12	384	89	192	38.44	2.06	52	384	9.46	38	384	32	192	89.59	2.91
0 + H2O2	31	384	80	192	43.77	4.81	65	384	10.59	98	384	48	192	69.31	10.63
70+ H2O2	32	384	79	192	44.40	4.90	94	384	15.81	103	384	39	192	79.70	9.80

* *Cultures 210 uM Ni3S2 and 140, 210 Ni3S2/ H2O2 died.

**Nickel stock solution was not prepared immediately prior to dosing.

Table 12: Nickel Subsulfide/Experiment 2

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	115	384	100.00	83	294	190
70	124	384	93.75	30	177	134
140	143	384	81.93	42	219	215
160	194	384	56.63	51	513	67
0 + H2O2	124	384	93.75	78	225	154
40 + H2O2	140	384	83.69	64	326	215
70 + H2O2	139	384	84.28	84	318	281
100 + H2O2	181	384	62.38	57	255	82

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	16	384	41	192	77.20	1.38	54	384	4.91	28	384	58	192	59.85	3.16
70	7	384	35	192	85.11	0.54	39	384	3.15	21	384	59	192	59.00	2.38
140	11	384	35	192	85.11	0.85	54	384	4.45	42	384	51	192	66.28	4.37
160	13	384	61	191	57.07	1.51	112	384	15.11	22	384	43	192	74.81	1.97
0 + H2O2	19	383	31	192	91.18	1.40	52	383	4.00	28	384	48	192	69.31	2.73
40 + H2O2	16	384	36	192	83.70	1.27	75	384	6.49	43	384	48	192	69.31	4.28
70 + H2O2	20	382	38	191	80.73	1.67	70	381	6.29	58	384	44	192	73.67	5.56
100 + H2O2	16	384	47	192	70.37	1.51	64	367	6.81	30	384	30	192	92.81	2.19

Table 13: Nickel Subsulfide/Experiment 3

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	90	384	100.00	97	217	183
140	139	384	70.04	56	165	63
250	185	384	50.34	34	320	52
300	204	384	43.60	43	249	98
0 + H2O2	108	384	87.43	129	363	206
140 + H2O2	131	384	74.13	101	251	193
180 + H2O2	206	384	42.93	52	296	98
220 + H2O2	215	384	39.98	47	333	146

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	19	384	40	192	78.43	1.62	41	383	3.61	32	384	46	192	71.44	3.04
140	19	384	29	192	94.51	1.34	53	384	3.93	17	384	42	192	75.99	1.49
250	12	384	47	192	70.37	1.13	99	384	10.59	22	384	34	186	84.97	1.74
300	18	383	44	192	73.67	1.63	94	384	9.53	46	384	35	192	85.11	3.75
0 + H2O2	29	384	39	192	79.70	2.46	76	384	6.92	52	384	30	191	92.55	3.93
140 + H2O2	34	384	25	192	101.93	2.27	79	384	5.65	46	384	44	192	73.67	4.33
180 + H2O2	22	384	44	192	73.67	2.00	110	383	11.49	42	384	42	192	75.99	3.81
220 + H2O2	17	382	60	192	58.16	1.96	106	384	13.89	74	384	33	192	88.05	6.08

Table 14: Nickel Subsulfide/Experiment 4

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	75	384	100.00	57	211	163
250	174	384	48.47	6	145	112
300	327	384	9.84			
380	376	384	1.29			
0 + H2O2	111	384	76.00	53	225	232
180 + H2O2	228	384	31.92	41	196	81
220 + H2O2	268	384	22.02	20	251	67
280 + H2O2	296	384	15.94			

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	13	384	31	191	90.91	0.95	46	383	3.52	26	384	53	192	64.36	2.72
250	3	382	28	192	96.26	0.20	67	384	4.98	36	384	53	190	63.84	3.86
0 + H2O2	13	384	44	192	73.67	1.17	52	384	4.94	44	384	58	192	59.85	5.08
180 + H2O2	23	384	45	192	72.54	2.13	98	382	10.22	36	384	60	192	58.16	4.23
220 + H2O2	14	384	55	192	62.51	1.49	145	384	18.96	37	383	70	192	50.45	5.03

**Cultures 300, 380 uM Ni3S2, and 280 uM Ni3S2/H2O2 died.

Table 15: Nickel Subsulfide/Experiment 5

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	97	383	100.00	45	230	153
140	126	384	81.14	29	152	98
250	245	384	32.72			
300	347	384	7.38			
380	361	384	4.50			
0 + H2O2	101	384	97.25	57	266	140
140 + H2O2	172	384	58.48	60	198	105
180 + H2O2	226	383	38.41			
220 + H2O2	316	384	14.19			

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	9	384	39	191	79.44	0.75	44	383	3.84	31	384	37	192	82.33	2.56
140	9	384	26	192	99.97	0.59	45	384	3.12	28	384	29	192	94.51	2.00
0 + H2O2	11	384	43	192	74.81	0.97	49	384	4.56	27	384	42	192	75.99	2.40
140 + H2O2	21	384	37	192	82.33	1.71	65	384	5.63	36	384	37	192	82.33	2.99
180 + H2O2	32	384	47	192	70.37	3.09	111	384	12.12	35	384	25	192	101.93	2.34

** Cultures 250, 300, 380 uM Ni3S2, and 220 uM Ni3S2/H2O2 died.

Table 16: Nickel Hydroxide/Experiment 1

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	81	384	100.00	94	221	135
250	87	384	95.41	56	225	125
350	94	384	90.44	71	298	121
450	123	384	73.16	73	369	91
0 + H2O2	120	384	74.74	36	247	213
200 + H2O2	126	384	71.61	90	317	178
300 + H2O2	146	384	62.14	76	420	127
400 + H2O2	195	384	43.54	23	306	147

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	17	384	45	192	72.54	1.56	39	384	3.69	22	384	52	192	65.31	2.26
250	12	384	38	192	81.00	0.98	46	384	3.94	24	384	44	192	73.67	2.19
350	15	384	42	192	75.99	1.31	59	384	5.49	24	384	45	192	72.54	2.22
450	17	384	49	192	68.28	1.66	78	380	8.41	27	384	33	192	88.05	2.07
0 + H2O2	7	384	62	192	56.52	0.81	45	384	5.51	43	384	55	192	62.51	4.75
200 + H2O2	22	384	47	192	70.37	2.10	72	384	7.38	52	384	33	192	88.05	4.13
300 + H2O2	21	384	48	192	69.31	2.03	103	384	11.26	40	384	38	192	81.00	3.40
400 + H2O2	7	384	66	192	53.39	0.86	85	384	11.72	54	384	50	192	67.27	5.63

Table 17: Nickel Hydroxide/Experiment 2

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	72	384	100.00	134	447	
350	119	384	69.98	42	237	83
450	143	384	59.01	71	355	67
600	205	384	37.49	24	579	122
0 + H2O2	122	384	68.50	100	263	
300 + H2O2	149	384	56.55	45	336	111
400 + H2O2	197	384	39.87	14	180	78
600 + H2O2	233	384	29.85			

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	26	384	40	192	78.43	2.23	80	384	7.45						
350	13	384	35	192	85.11	1.01	67	383	5.65	25	384	35	191	84.85	1.98
450	25	383	36	192	83.70	2.02	106	372	10.02	19	384	50	192	67.27	1.89
600	7	384	80	190	43.25	1.06	138	384	25.74	53	384	49	192	68.28	5.44
0 + H2O2	31	384	34	192	86.56	2.43	76	383	6.39						
300 + H2O2	20	384	25	192	101.93	1.31	127	382	9.91	38	384	39	192	79.70	3.27
400 + H2O2	8	384	31	192	91.18	0.58	92	384	7.51	39	384	37	192	82.33	3.25

** Culture 600 uM Ni(OH)2/H2O2 died.

**Control and 0 + H2O2 hprt plates were contaminated.

Table18: Nickel Hydroxide/Experiment 3

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	76	373	100.00	54	221	113
350	137	382	64.79	46	177	49
450	161	381	54.15	45	305	77
600	189	376	43.24	61	365	33
0 + H2O2	83	384	96.29	47	260	89
300 + H2O2	156	384	56.62	56	150	53
400 + H2O2	201	384	40.69	51	286	91
500 + H2O2	220	384	35.01	53	313	31

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	11	384	38	192	81.00	0.90	43	383	3.68	22	384	40	192	78.43	1.88
350	15	384	35	192	85.11	1.17	55	384	4.54	15	384	40	192	78.43	1.27
450	17	384	37	192	82.33	1.37	102	384	9.38	25	384	46	192	71.44	2.36
600	23	384	52	192	65.31	2.36	118	384	14.05	17	384	32	192	89.59	1.26
0 + H2O2	10	384	38	192	81.00	0.81	52	383	4.50	23	384	26	192	99.97	1.54
300 + H2O2	19	384	41	192	77.20	1.64	49	384	4.42	19	384	38	192	81.00	1.57
400 + H2O2	22	384	47	192	70.37	2.10	108	384	11.73	37	384	49	192	68.28	3.71
500 + H2O2	24	384	54	192	63.43	2.54	120	381	14.91	18	384	38	192	81.00	1.48

Table 19: Nickel Metal Powder/Experiment 1

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	72	384	100.00	40	166	69
400	89	384	87.34	32	186	230
600	143	384	59.01	55	158	77
800	181	384	44.93	34	132	63
1000	231	384	30.36	43	265	71
0 + H2O2	123	384	68.01	77	185	53
400 + H2O2	145	384	58.18	73	201	108
600 + H2O2	245	382	26.53	33	132	40
800 + H2O2	290	383	16.77			

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	8	384	40	192	78.43	0.67	32	384	2.77	12	384	48	192	69.31	1.15
400	6	384	52	192	65.31	0.60	34	384	3.55	43	365	46	192	71.44	4.39
600	16	382	49	192	68.28	1.57	40	348	4.47	27	384	36	192	83.70	2.18
800	18	384	28	192	96.26	1.25	66	383	4.91	32	384	30	192	92.81	2.34
1000	21	384	58	192	59.85	2.35	113	384	14.56	41	384	45	192	72.54	3.89
0 + H2O2	22	384	40	192	78.43	1.88	51	384	4.54	16	356	33	192	88.05	1.31
400 + H2O2	22	384	47	192	70.37	2.10	57	381	5.76	29	384	54	192	63.43	3.10
600 + H2O2	17	384	37	192	82.33	1.37	92	384	8.32	26	384	47	192	70.37	2.49

** Culture 800 uM Nickel Powder/H2O2 died.

Table 20: Nickel Metal Powder/Experiment 2

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	96	384	100.00	69	121	85
400	98	384	98.51	73	192	96
800	105	384	93.54	43	220	85
1000	172	384	57.94			
0 + H2O2	101	384	96.34	65	229	99
400 + H2O2	166	384	60.50	51	184	109
500 + H2O2	121	384	83.31	114	195	88
600 + H2O2	133	384	76.48	139	241	195

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	18	384	24	192	103.97	1.15	31	384	2.02	19	384	32	192	89.59	1.42
400	17	384	31	192	91.18	1.24	43	384	3.26	22	384	31	192	91.18	1.62
800	11	384	29	192	94.51	0.77	53	384	3.93	24	384	23	192	106.10	1.52
0 + H2O2	14	384	37	192	82.33	1.13	47	384	3.96	24	384	29	192	94.51	1.71
400 + H2O2	20	384	29	192	94.51	1.41	67	384	5.07	42	384	28	192	96.26	3.01
500 + H2O2	30	383	32	192	89.59	2.28	50	383	3.90	24	384	31	192	91.18	1.77
600 + H2O2	37	384	36	192	83.70	3.03	62	384	5.26	47	383	41	192	77.20	4.24

** Culture 1000 uM nickel powder was contaminated.

Table 21: Nickel Metal Powder/Experiment 3

Test Compd. (uM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	87	384	100.00	93	176	127
400	98	384	91.98	55	243	205
800	198	384	44.61	112	258	74
1000	266	384	24.73	24	277	57
0 + H2O2	109	384	84.82	137	146	85
400 + H2O2	205	384	42.27	47	280	97
500 + H2O2	194	384	45.99	61	218	100
600 + H2O2	195	384	45.64	94	205	111

Test Compd. (uM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	20	384	34	192	86.56	1.54	37	384	2.93	25	384	39	192	79.70	2.11
400	14	384	30	192	92.81	1.00	58	384	4.41	30	384	64	191	54.67	3.72
800	42	384	48	192	69.31	4.18	90	384	9.63	35	384	34	191	86.30	2.77
1000	16	384	51	192	66.28	1.61	150	384	18.68	41	384	44	192	73.67	3.83
0 + H2O2	31	384	40	192	78.43	2.68	33	384	2.86	19	384	42	192	75.99	1.67
400 + H2O2	13	384	76	192	46.34	1.86	71	384	11.03	34	384	57	192	60.72	3.82
500 + H2O2	25	384	42	192	75.99	2.21	82	384	7.90	42	384	39	192	79.70	3.63
600 + H2O2	34	384	50	192	67.27	3.45	70	384	7.48	44	384	43	192	74.81	4.07

Table 22: Nickel Oxide/Experiment 1

Test Compd. (mM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	81	382	100.00	75	213	128
16	82	384	99.55	75	159	137
32	90	384	93.54	44	324	81
50	82	384	99.55	32	353	105
0 + H2O2	137	384	66.45	145	210	97
16 + H2O2	117	378	75.61	61	329	155
32 + H2O2	126	384	71.85	31	259	167
50 + H2O2	115	384	77.74	14	369	34

Test Compd. (mM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	15	384	39	192	79.70	1.25	41	384	3.54	22	384	48	192	69.31	2.13
16	14	384	44	192	73.67	1.26	29	384	2.66	24	384	47	192	70.37	2.29
32	9	384	43	192	74.81	0.79	61	384	5.78	13	384	58	192	59.85	1.44
50	7	384	34	192	86.56	0.53	71	384	5.90	23	384	33	192	88.05	1.75
0 + H2O2	44	384	36	192	83.70	3.63	62	384	5.26	27	384	43	192	74.81	2.44
16 + H2O2	16	382	39	192	79.70	1.34	79	383	7.25	39	383	40	192	78.43	3.42
32 + H2O2	9	384	37	192	82.33	0.72	69	384	6.01	46	384	37	192	82.33	3.87
50 + H2O2	4	384	34	192	86.56	0.30	92	384	7.91	10	384	32	192	89.59	0.74

Table 23: Nickel Oxide/Experiment 2

Test Compd. (mM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	77	384	100.00	72	261	124
16	84	384	94.59	36	236	
32	105	384	80.70	30	149	43
50	89	384	90.99	14	316	
0 + H2O2	121	384	71.87	85	517	
16 + H2O2	119	384	72.91	72	312	125
32 + H2O2	130	384	67.41			
50 + H2O2	107	384	79.52	100	229	81

Test Compd. (mM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	17	384	29	190	93.99	1.20	58	384	4.36	18	384	60	192	58.16	2.06
16	8	384	36	192	83.70	0.63	50	384	4.17						
32	11	384	19	192	115.65	0.63	51	384	3.08	9	384	50	192	67.27	0.88
50	3	384	43	192	74.81	0.26	61	384	5.78						
0 + H2O2	22	384	43	191	74.55	1.98	113	376	11.99						
16 + H2O2	20	384	38	192	81.00	1.65	79	383	7.13	28	384	51	192	66.28	2.86
50 + H2O2	24	384	41	192	77.20	2.09	52	378	4.79	22	384	34	192	86.56	1.70

** Culture 32 mM NiO/H2O2 was contaminated.

**Hprt plates 16 mM NiO/H2O2 and 0 + H2O2 were contaminated.

Table 24: Nickel Oxide/Experiment 3

Test Compd. (mM)	Toxicity			Surviving Mutants		
	-wells	T wells	% Survival	tk	tk-slow	hprt
0	84	384	100.00	129	173	107
16	90	384	95.46	135	159	101
32	97	384	90.53	33	171	124
50	103	384	86.58	90	166	84
0 + H2O2	104	384	85.95	68	154	49
16 + H2O2	86	384	98.45	189	143	74
32 + H2O2	125	384	73.85	28	175	25
50 + H2O2	121	384	75.99	40	163	46

Test Compd. (mM)	tk-Normal						tk- Slow			hprt					
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)
0	30	768	30	192	92.81	2.15	40	767	2.89	23	383	34	192	86.56	1.79
16	34	768	28	192	96.26	2.35	40	768	2.78	22	384	36	192	83.70	1.76
32	8	768	35	192	85.11	0.62	40	768	3.14	17	384	71	192	49.74	2.28
50	23	768	33	192	88.05	1.73	42	768	3.19	19	384	40	192	78.43	1.62
0 + H2O2	18	768	32	192	89.59	1.32	39	748	2.99	13	384	31	192	91.18	0.94
16 + H2O2	42	768	33	192	88.05	3.19	32	768	2.42	16	381	35	192	85.11	1.26
32 + H2O2	8	768	36	192	83.70	0.63	49	768	3.94	7	384	37	192	82.33	0.56
50 + H2O2	12	768	32	192	89.59	0.88	47	759	3.57	11	384	46	192	71.44	1.02

**For tk determination eight plates were seeded at 20,000 cells/well.

Table 25: Nickel Oxide/Experiment 4

Test Compd.	Toxicity			Surviving Mutants	
(mM)	-wells	T wells	% Survival	tk	tk-slow
0	80	384	100.00	77	195
16	81	384	99.21	159	251
32	83	384	97.65	56	171
50	83	384	97.65	30	280
0 + H2O2	117	384	75.77	54	149
16 + H2O2	104	384	83.27	114	300
32 + H2O2	96	384	88.38	124	158
50 + H2O2	132	384	68.08	66	179

Test Compd.	tk-Normal						tk- Slow		
(mM)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)
0	18	768	30	192	92.81	1.28	45	768	3.25
16	32	768	39	192	79.70	2.67	50	768	4.22
32	12	768	37	192	82.33	0.96	36	768	2.92
50	7	768	33	192	88.05	0.52	62	768	4.78
0 + H2O2	14	768	41	192	77.20	1.19	38	768	3.29
16 + H2O2	27	768	40	192	78.43	2.28	69	768	6.00
32 + H2O2	30	768	35	192	85.11	2.34	38	768	2.98
50 + H2O2	18	768	44	192	73.67	1.61	48	768	4.38

**No hprt plating performed. For tk determination, eight plates were seeded at 20,000 cells/well.

Table 26: Radical Scavenger/Experiment 1

Test Compd. (uM)	Toxicity			Surviving Mutants	
	-wells	T wells	% Survival	tk	tk-slow
0	86	384	100.00	91	178
O + Vit E.	82	384	103.18	101	192
NiSO4	371	384	2.30	5	5
NiSO4 + Vit. E	377	384	1.23	2	3
Ni3S2	277	384	21.83	42	110
Ni3S2 + Vit. E	278	384	21.59	30	151
Ni(OH)2	256	384	27.10	27	90
Ni(OH)2 + Vit. E	274	384	22.56	23	79
Ni Metal	257	384	26.84	39	139
Ni Metal + Vit. E	281	384	20.87	32	126

Test Compd. (uM)	tk-Normal						tk- Slow		
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)
0	21	384	30	192	92.81	1.51	40	384	2.96
O + Vit E.	21	384	34	191	86.30	1.63	39	384	3.10
NiSO4	45	384	37	192	82.33	3.78	44	384	3.70
NiSO4 + Vit. E	39	384	31	192	91.18	2.94	46	383	3.51
Ni3S2	37	384	39	192	79.70	3.18	90	384	8.38
Ni3S2 + Vit. E	31	384	32	192	89.59	2.35	131	384	11.64
Ni(OH)2	24	384	28	192	96.26	1.68	74	384	5.56
Ni(OH)2 + Vit. E	25	384	26	192	99.97	1.68	80	384	5.84
Ni Metal	26	384	45	192	72.54	2.42	85	384	8.62
Ni Metal + Vit. E	29	384	42	192	75.99	2.58	101	383	10.07

Table 27: Radical Scavenger/Experiment 2

Test Compd. (uM)	Toxicity			Surviving Mutants	
	-wells	T wells	% Survival	tk	tk-slow
0	53	384	100.00	64	220
O + Vit E.	72	384	84.53	38	175
NiSO4	281	384	15.77	28	91
NiSO4 + Vit. E	312	384	10.49	10	71
Ni3S2	147	384	48.49	40	166
Ni3S2 + Vit. E	116	384	60.45	45	255
Ni(OH)2	154	384	46.14	48	235
Ni(OH)2 + Vit. E	179	384	38.54	38	198
Ni Metal	354	384	4.11		
Ni Metal + Vit. E	287	384	14.70		

Test Compd. (uM)	tk-Normal						tk-Slow		
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)
0	13	384	38	192	81.00	1.06	43	384	3.67
O + Vit E.	9	384	39	192	79.70	0.74	40	384	3.45
NiSO4	35	384	38	191	80.73	2.96	102	382	9.62
NiSO4 + Vit. E	17	384	43	192	74.81	1.51	110	384	11.28
Ni3S2	18	384	33	192	88.05	1.36	70	384	5.71
Ni3S2 + Vit. E	15	384	39	191	79.44	1.25	77	384	7.04
Ni(OH)2	19	384	44	192	73.67	1.72	85	384	8.49
Ni(OH)2 + Vit. E	20	384	37	190	81.81	1.63	94	384	8.58

**Cultures nickel metal powder +/- Vitamin E died.

Table 28: Radical Scavenger/Experiment 3

Test Compd. (uM)	Toxicity			Surviving Mutants	
	-wells	T wells	% Survival	tk	tk-slow
0	59	384	100.00	138	253
O + Vit E.	85	384	80.51	93	264
NiSO4	312	384	11.09	23	67
NiSO4 + Vit. E	305	384	12.30	22	101
Ni3S2	122	384	61.22	39	237
Ni3S2 + Vit. E	136	384	55.42	26	356
Ni(OH)2	174	384	42.26	101	339
Ni(OH)2 + Vit. E	185	384	38.99	30	275
Ni Metal	246	382	23.50	33	263
Ni Metal + Vit. E	226	384	28.30	73	311

Test Compd. (uM)	tk-Normal						tk-Slow		
	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)
0	24	384	47	192	70.37	2.29	43	384	4.22
O + Vit E.	20	384	48	192	69.31	1.93	54	384	5.47
NiSO4	39	384	41	192	77.20	3.47	103	384	10.11
NiSO4 + Vit. E	34	384	40	192	78.43	2.96	134	384	13.68
Ni3S2	14	384	34	192	86.56	1.07	77	384	6.46
Ni3S2 + Vit. E	8	384	51	192	66.28	0.79	95	384	10.72
Ni(OH)2	39	384	50	192	67.27	3.98	115	381	13.35
Ni(OH)2 + Vit. E	19	384	26	192	99.97	1.27	144	384	11.75
Ni Metal	23	384	52	192	65.31	2.36	148	384	18.63
Ni Metal + Vit. E	38	384	57	192	60.72	4.29	138	384	18.33

Table 29: Summary for Radical Scavenger Experiment Data: Induced Mutant Fraction (IMF) for Slow-growing tk-/- Mutants

Test Compound	IMF (10-6) Experiment 1	IMF (10-6) Experiment 2	IMF (10-6) Experiment 3
NiSO4	0.74	5.95	5.89
NiSO4 + Vit. E.	0.41	7.83	8.21
Ni3S2	5.42	2.04	2.24
Ni3S2 + Vit. E.	8.54	3.59	5.25
Ni(OH)2	2.6	4.82	9.13
Ni(OH)2 + Vit. E.	2.74	5.13	6.28
Ni Metal	5.66	ND	14.41
Ni Metal + Vit. E.	6.97	ND	12.86

**ND indicates no data

Table 30: Experiments with Nickel Sulfate-treated TK6 Cells Plated in the Presence of TFT after 4.0 and 9.0 Doubings

Test Compd.	Toxicity			Surviving Mutants	
(60 uM)	-wells	T wells	% Survival	tk	tk-slow
Control 1	104	384	100.00	30	361
Treatment 1	125	384	85.92	29	386
Control 2	95	384	100.00	43	388
Treatment 2	113	384	87.58	53	378
Control 3	99	384	100.00	67	259
Treatment 3	112	384	90.90	87	382
Control 4	90	384	100.00	47	372
Treatment 4	129	384	75.17	27	425

Plated after 4.0 doublings

Test Compd.	tk-Normal						tk- Slow		
(60 uM)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)
Control 1	6	384	40	192	78.43	0.50	66	384	6.01
Treatment 1	7	384	37	192	82.33	0.56	84	384	7.50
Control 2	10	384	30	192	92.81	0.71	82	384	6.47
Treatment 2	14	384	30	192	92.81	1.00	90	384	7.19
Control 3	15	384	32	192	89.59	1.11	55	384	4.31
Treatment 3	21	384	33	192	88.05	1.60	84	384	7.01
Control 4	10	384	35	192	85.11	0.78	73	384	6.19
Treatment 4	8	384	34	192	86.56	0.61	107	384	9.43

Plated after 9.0 doublings

Test Compd.	tk-Normal						tk- Slow		
(60 uM)	+wells	T wells	PE-wells	PE T wells	PE (%)	MF(10E-6)	+wells	T wells	MF(10E-6)
Control 1	13	384	40	192	78.43	1.10	76	384	7.03
Treatment 1	23	384	35	192	85.11	1.81	75	384	6.38
Control 2	23	384	36	192	83.70	1.84	64	384	5.45
Treatment 2	7	384	27	192	98.08	0.47	82	384	6.12
Control 3	18	384	24	192	103.97	1.15	73	384	5.07
Treatment 3	17	384	34	192	86.56	1.31	99	384	8.61
Control 4	19	384	25	192	101.93	1.24	72	384	5.09
Treatment 4	24	384	25	192	101.93	1.58	78	384	5.57

Table 31: Statistical Significance (*) and Corresponding p-values for the Induction of Slow-growing tk-/- Mutants at Several Nickel Concentrations

Nickel Sulfate (uM)	p-value	Nickel Subsulfide (uM)	p-value	Nickel Hydroxide (uM)	p-value
0		0		0	
20	0.3287	70	0.5507	250	0.7805
40	0.2776	140	0.7794	350	0.3166
60	0.4299	160*	0.0000	450*	0.0474
95	0.4283	250*	0.0340	600*	0.0013
145*	0.0000	300*	0.0000		
175	0.1416				

Nickel Metal Powder (uM)	p-value	Nickel Oxide (mM)	p-value
0		0	
400	0.2749	16	1.1068
600	0.0641	32	0.7470
800*	0.0125	50	0.4598
1000*	0.0000		

Note: Signficance was determined by two-sided, normal z-tests, p-value <0.05

Mutation Assay Exposure Summary

I. Cell Preparation

CHAT Selection (48 hrs)
Recovery in THC (24 hrs)
Cell Accumulation (24-48 hrs)

II. Exposure

Nickel Compounds (24 hrs)
Hydrogen Peroxide (16 hrs)
Vitamin E (24 hrs)

III. Plate for Toxicity →

III. Phenotypic Expression Period

(Removal of Nickel by Centrifugation or Daily Dilutions)
~ 4.0 doublings for tk expression
~ 9.0 doublings for hprt expression

IV. Plate for Mutagenicity

Selection for tk Mutants
(TFT)
↓
Score
Normal-growing *tk-/-*
re-feed w/TFT
(Day 10)
↓
Score
Slow-growing *tk-/-*
(Day 20)

Selection for hprt mutants
(6-TG)
↓
Score
hprt-
(Day 14)

Score for Survival
(Day 14)

Figure 1: Mutation Assay Exposure Summary

Nickel Sulfate

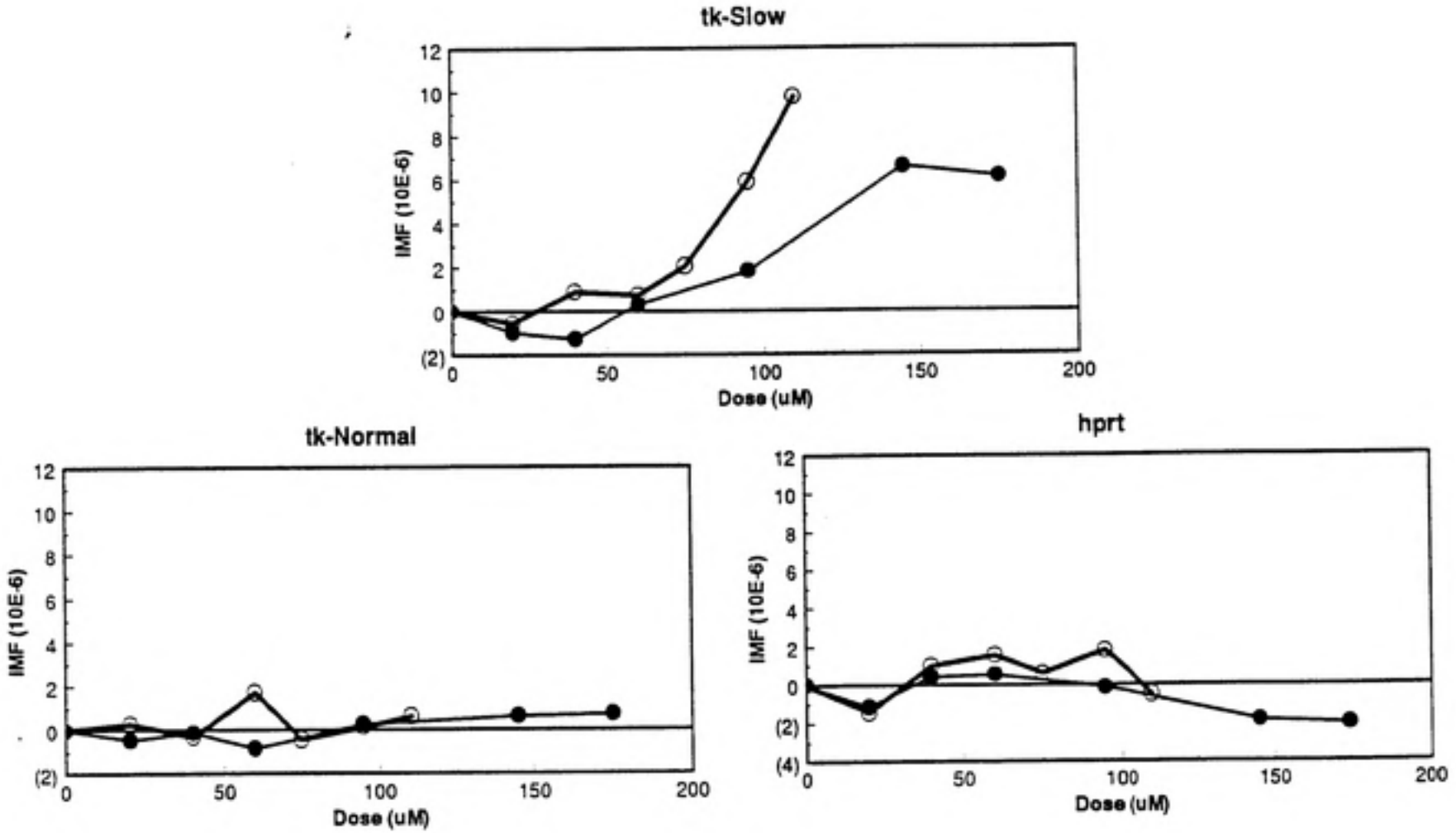

Figure 2: Induced mutant fraction (IMF) for TK6 cells exposed to nickel sulfate in the absence (closed circles) and presence (open circles) of H2O2. Mutant frequency values are averaged across experiments.

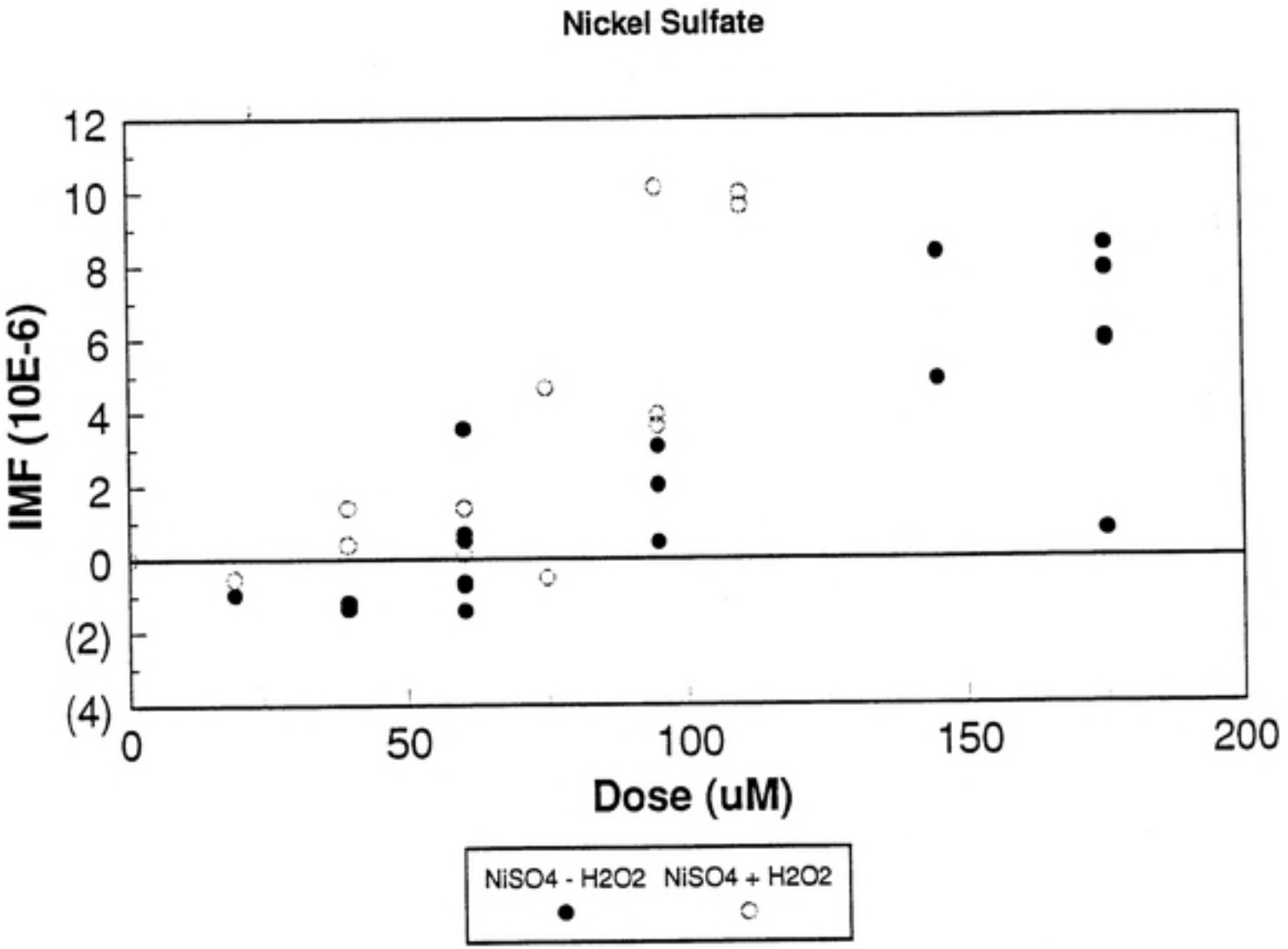

Figure 3: Induced mutant fraction (IMF) for tk-slow growth mutants in TK6 cells exposed to nickel sulfate +/- H2O2.

Nickel Subsulfide

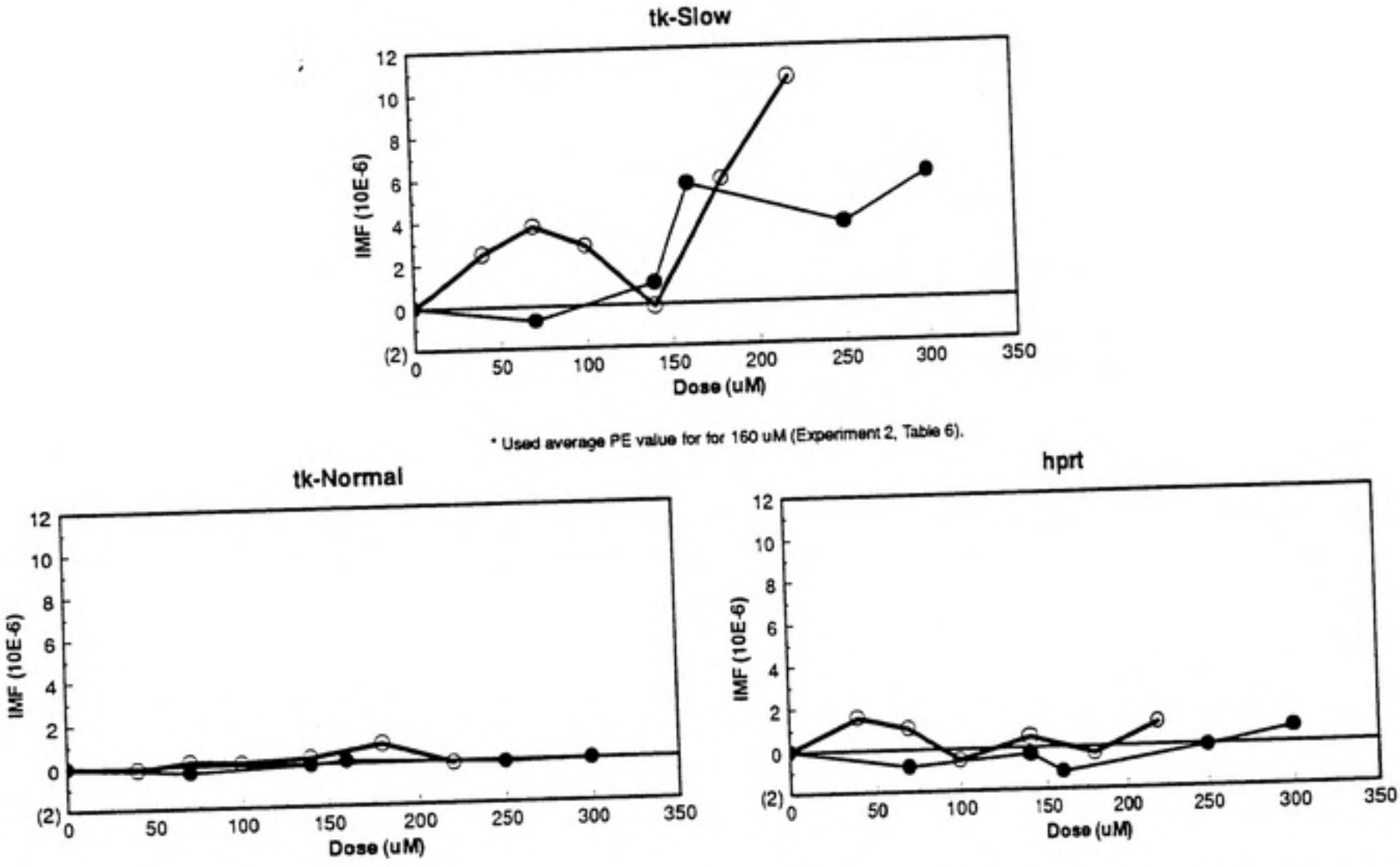

Figure 4: Induced mutant fraction (IMF) for TK6 cells exposed to nickel subsulfide in the absence (closed circles) and presence (open circles) of H2O2. Mutant frequency values are averaged across experiments.

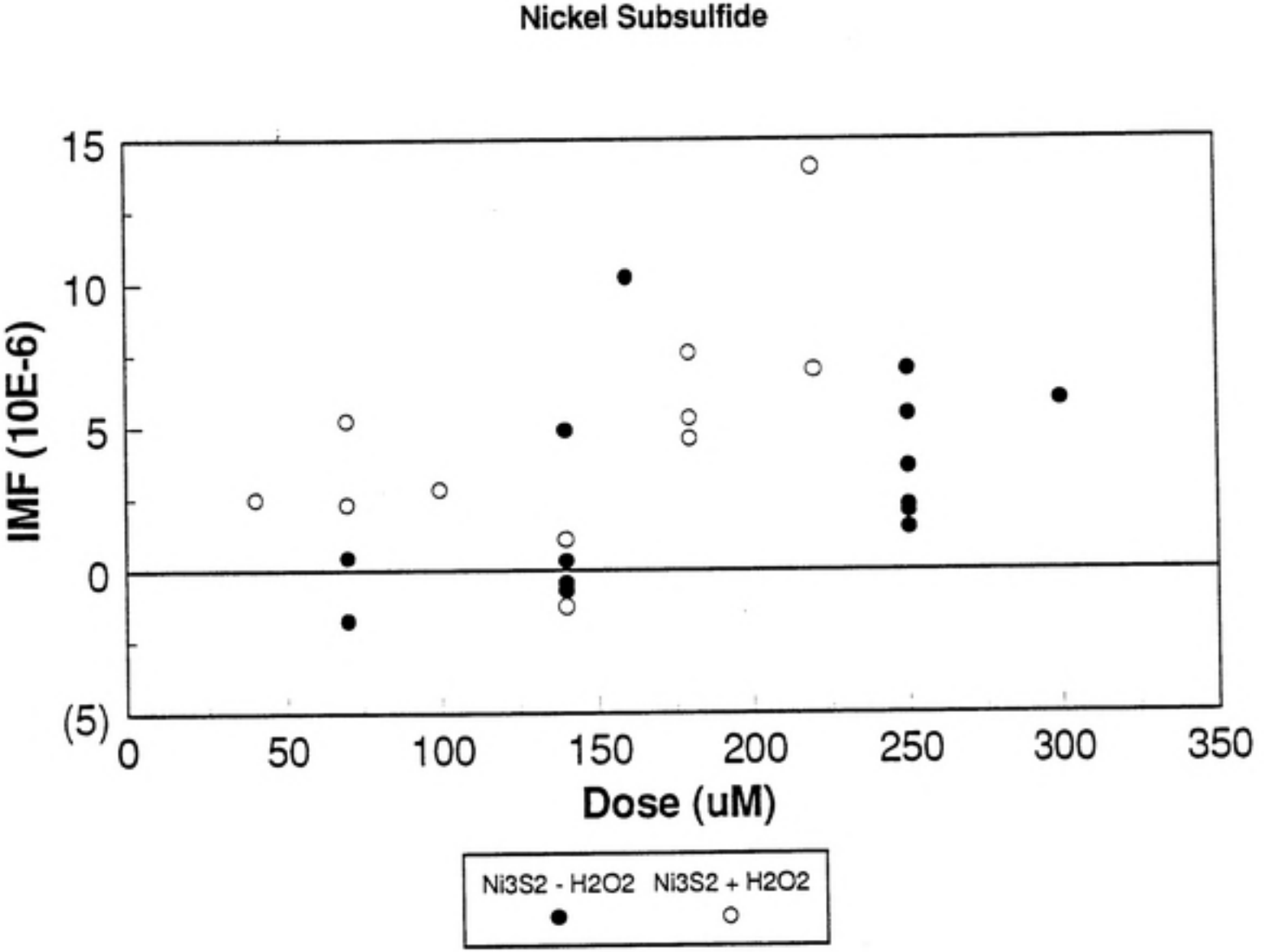

Figure 5: Induced mutant fraction (IMF) for tk-slow growth mutants in TK6 cells exposed to nickel subsulfide +/- H2O2.

Nickel Hydroxide

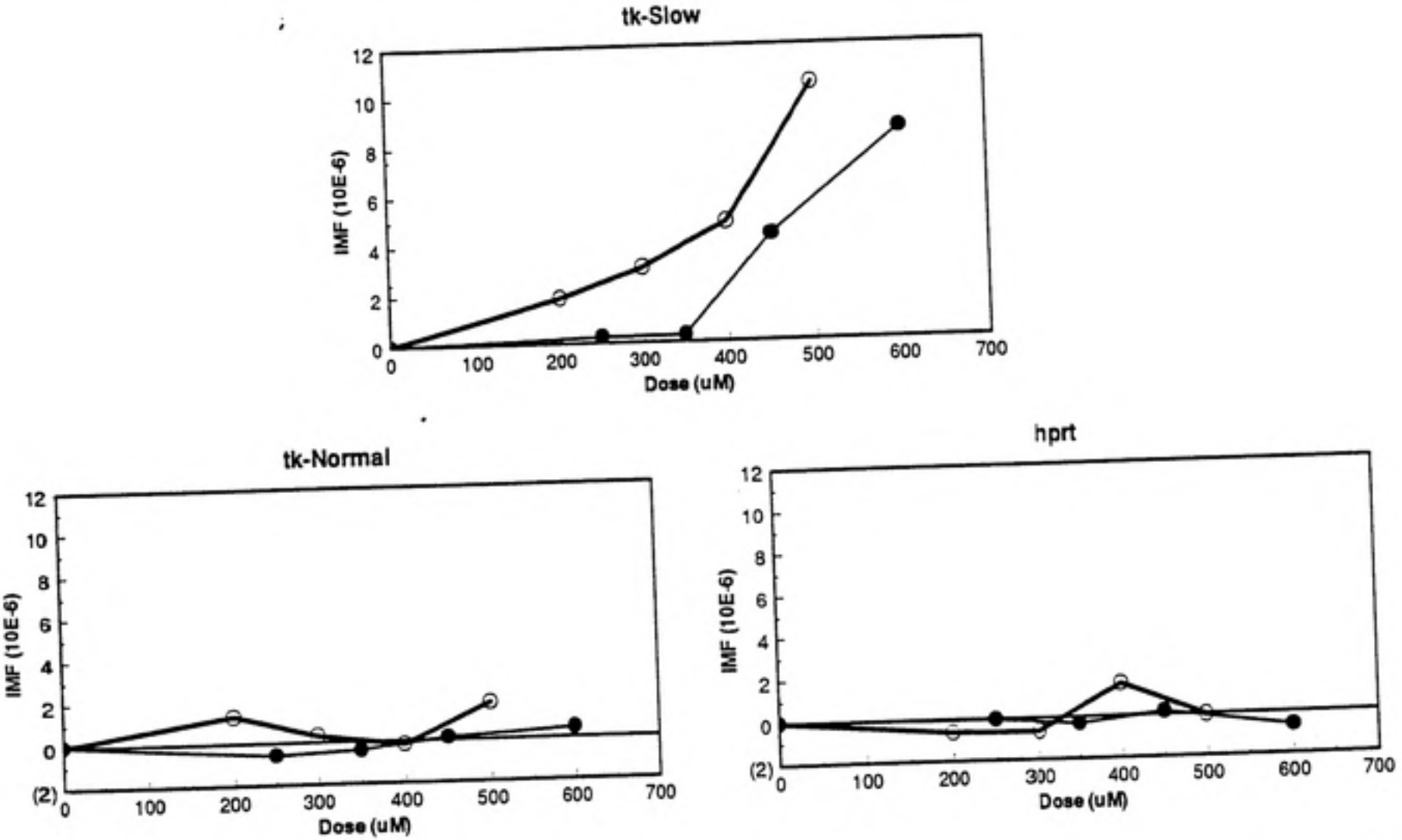

Figure 6: linduced mutant fraction (IMF) for TK6 cells exposed to nickel hydroxide in the absence (closed circles) and presence (open circles) of H2O2. Mutant frequency values are averaged over experiments.

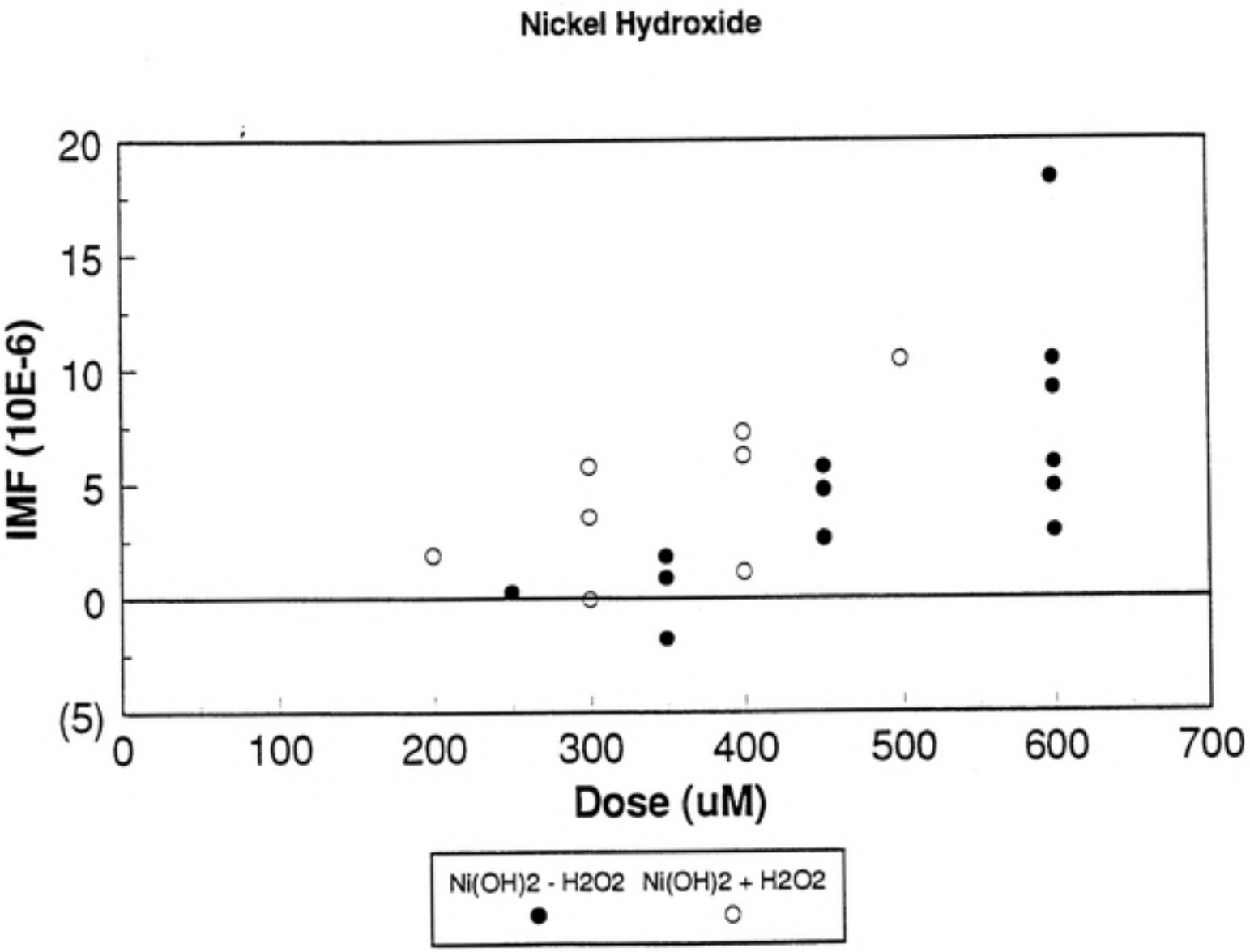

Figure 7: Induced mutant fraction (IMF) for tk-slow growth mutants in TK6 cells exposed to nickel hydroxide +/- H2O2.

Nickel Metal Powder

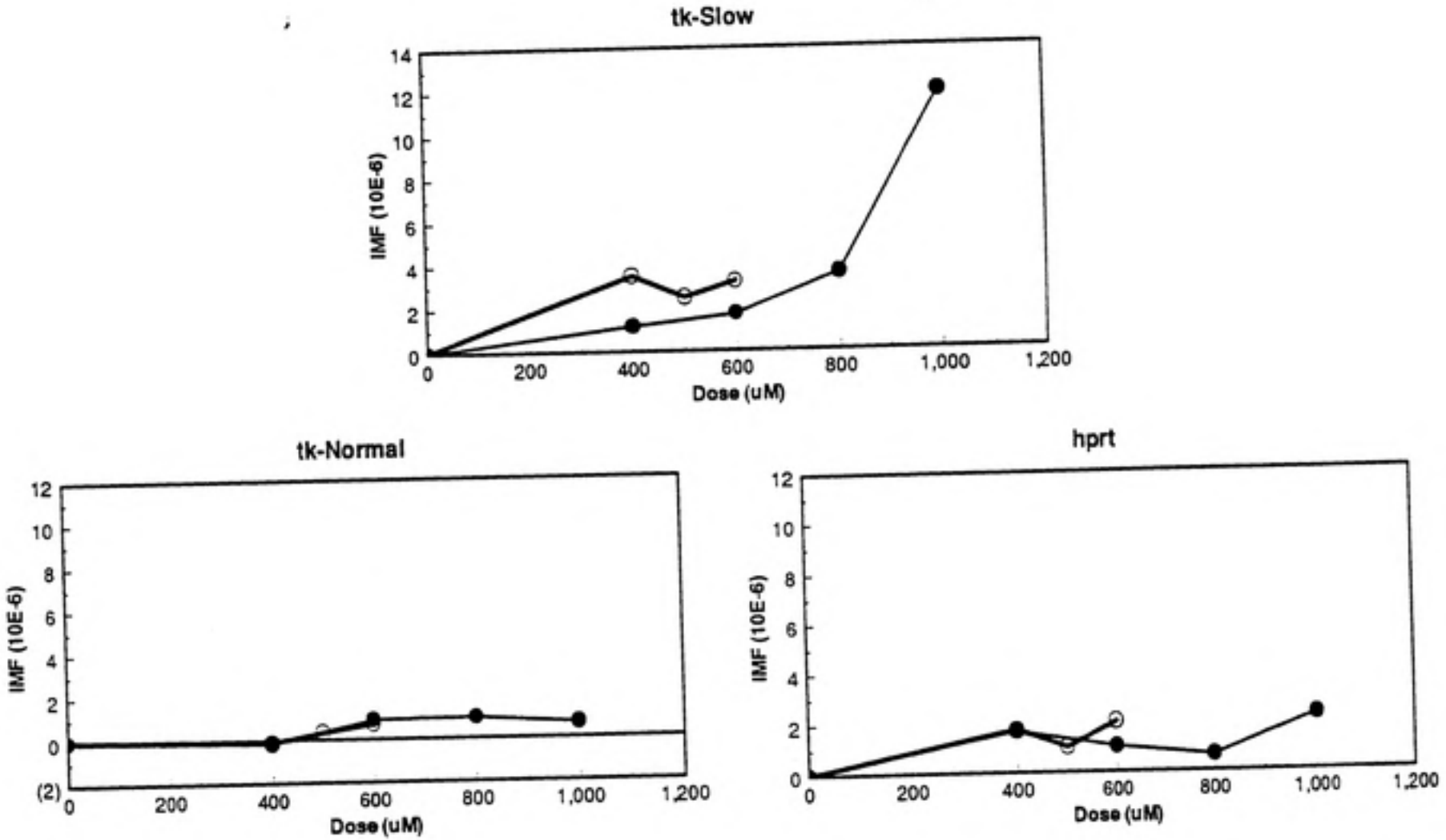

Figure 8: Induced mutant fraction (IMF) for TK6 cells exposed to nickel metal powder in the absence (closed circles) and presence (open circles) of H2O2. Mutant frequency values are averaged over experiments.

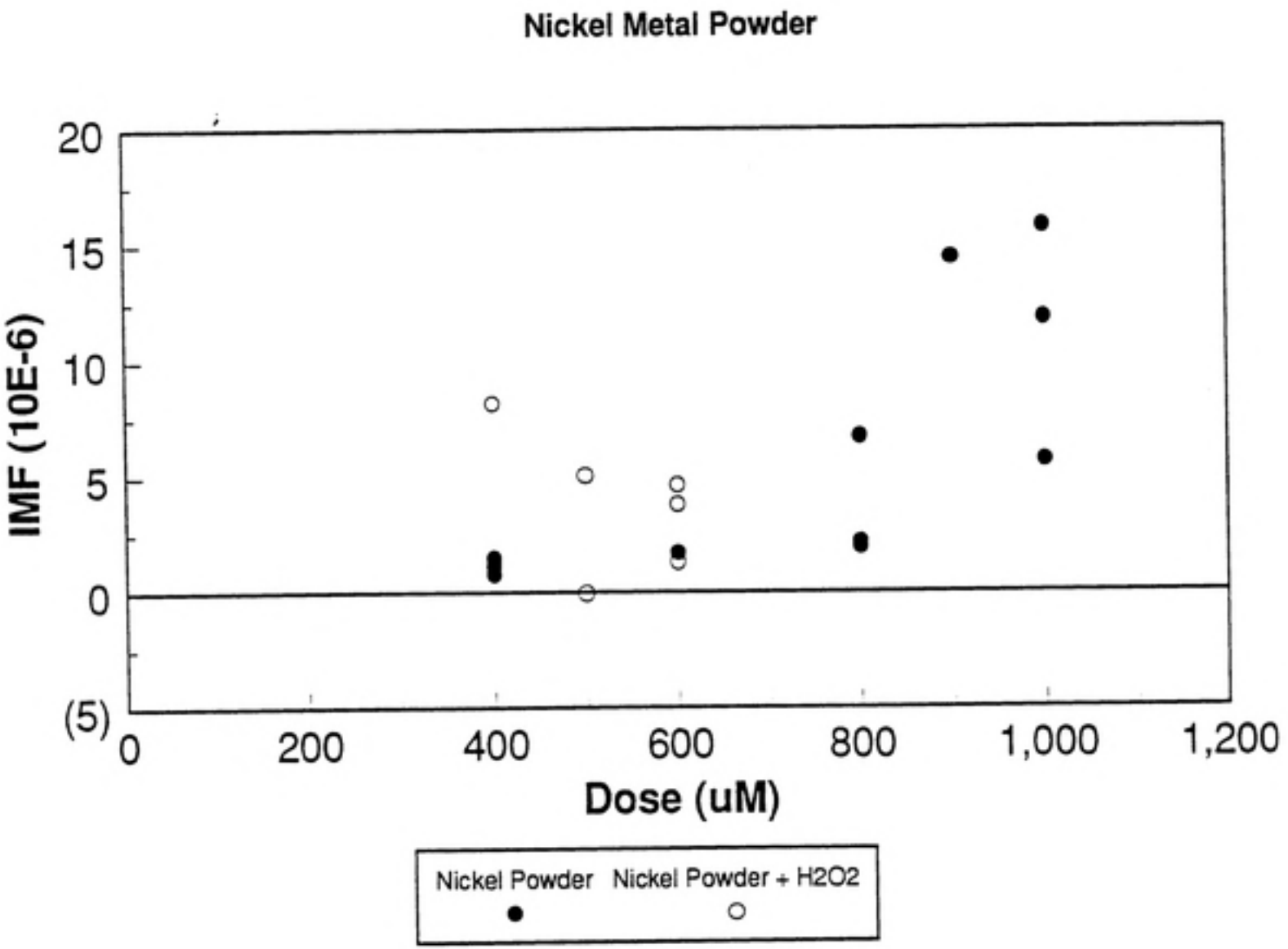

Figure 9: Induced mutant fraction (IMF) for tk-slow growth mutants in TK6 cells exposed to nickel metal powder +/- H2O2.

Nickel Oxide

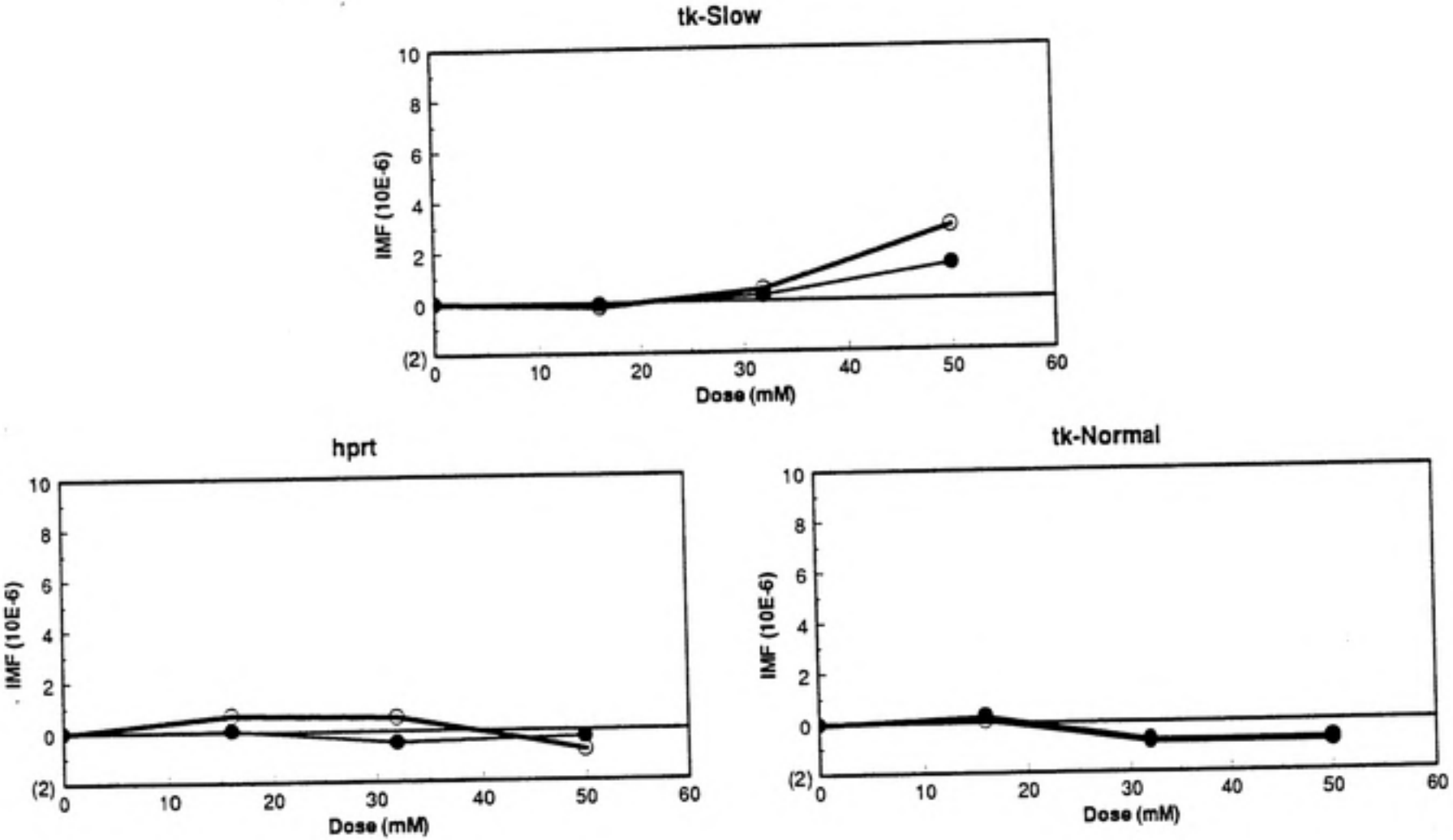

Figure 10: Induced mutant fraction (IMF) for TK6 cells exposed to nickel oxide in the absence (closed circles) and presence (open circles) of H2O2. Mutant frequency values are averaged across experiments.